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Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline

This document provides guidance related to proper and safe biological specimen collection and nucleic acid isolation and purification. These topics include methods of collection, recommended storage and transport conditions, and available nucleic acid purification technologies for each specimen/nucleic acid type.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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Abstract

Molecular methods involving the hybridization or enzymatic amplification of nucleic acids require the isolation and purification of these nucleic acids from a variety of biological specimens and microorganisms contained in these specimens. CLSI document MM13-A—*Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline* addresses topics that relate to proper and safe biological specimen collection and nucleic acid isolation and purification. These topics include methods of collection, recommended storage and transport conditions, and available nucleic acid purification technologies for each specimen/nucleic acid type.

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Contents

Foreword

This guideline was developed in response to the exponential growth of the numbers and types of molecular tests performed worldwide and the need to standardize specimen collection and preparation parameters for these tests. Until recently, molecular test methods routinely used by clinical laboratories were largely limited to detection and/or quantitation of viruses or bacteria in only a few specimen types. The accelerated identification of molecular lesions in neoplastic cells; the discoveries of genotypic variations that correlate to disease states; the significance of gene transcription as indicator of disease or response to therapy; and the commercialization of molecular diagnostic tests for routine use have contributed to both the variety of specimens used and the analytical techniques employed.

MM13 is part of a series of guidelines that address the needs of molecular diagnostic testing laboratories or other laboratories that apply molecular methods to the study of nucleic acids in human samples or specimens. Because of the variety of specimen types used, the many variables that can affect test results, and, indeed, the variety of test methodologies employed in molecular laboratories, the Subcommittee on Sample Collection and Handling for Molecular Test Methods concluded that it would be advantageous to provide guidelines that address the general principles for minimizing or eliminating preanalytical variables for all types of molecular tests and for all types of samples. This guideline should increase awareness of the sample handling factors that affect molecular testing results and promote standardization of the preanalytical phase of these test methods.

Key Words

Degradation, disruption, DNA, enrichment, expression, homogenization, RNA

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1 Scope

This guideline describes general principles for ensuring optimal specimen collection, transport, storage, and nucleic acid isolation for molecular diagnostic test methods. It is intended for all healthcare professionals responsible for obtaining and transporting specimens from patients or preparing samples for subsequent molecular tests. It is also intended for manufacturers of specimen collection devices and sample preparation reagents, kits, and instrumentation.

In addition, this document describes specimen collection and transport devices, and sample preparation methods. Optimal storage conditions and special precautions for molecular methods are described. While this document is intended for diagnostic testing, it is possible that the principles described here may apply to other areas.

NOTE: Measurand quality assessment should be undertaken at the level of the test method during method validation and is beyond the scope of this guideline. For information related to measurand (purified DNA or RNA) quality assessment, refer to the most current editions of the following CLSI/NCCLS documents: MM3—*Molecular Diagnostic Methods for Infectious Diseases*; MM5—*Nucleic Acid Amplification Assays for Molecular Hematopathology*; and/or MM9—*Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine*.

2 Introduction

The expanding discipline of molecular pathology is characterized by the rapid introduction of new markers for disease and technologies for their detection. Furthermore, nucleic acid targets must be isolated from a wide variety of clinical specimens, and the quality and quantity of extracted target is highly affected by specimen collection, handling, and choice of extraction method.

Molecular biological techniques developed in the last decade detect the presence or quantity of viruses; determine the family, genus, or species of microorganisms; or determine the viral genotype. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay-Sachs disease to name a few examples. Because of their inherent instability, the measurement of intracellular RNA targets has lagged behind DNA targets in contributing to patient management. Nevertheless, recurrence of hematological malignancies are increasingly detected by quantitation of gene translocation fusion transcripts characterizing the disease and appearing during or subsequent to treatment. The labile nature of RNA in particular has made standardization of these tests difficult or impossible. Furthermore, a negative result in a poorly handled sample may have been due to target degradation rather than the absence of disease.

The Subcommittee on Sample Collection and Handling for Molecular Test Methods recognizes the complex nature of the matrix of variables associated with specimen type, nucleic acid target, and compatibility of sample preparation methods with downstream test methodology. Accordingly, these guidelines are organized with these complexities in mind, and they have been designed for the laboratorian seeking a comprehensive, easy-to-use reference for molecular specimen handling. A chart is provided which allows the user to access specific information by cross-referencing specimen type with the nucleic acid target. Optimal conditions for transport and storage, as well as recommendations for nucleic acid extraction procedures, are provided when supported by published studies. In other cases, the

experience of committee members or recommendations from manufacturers of commercially available products is used.

Since molecular diagnostic methods can themselves be highly variable, the successful application of these techniques is well served by minimizing the preanalytical variables surrounding specimen acquisition, transport, storage, and processing. It is hoped that this guideline will further the standardization of preanalytical methods for the growing list of clinically valuable molecular diagnostic assays, with the important caveat that any of these methods may require optimization or qualification for test systems incorporated into clinical diagnostic testing. Laboratories and test developers are encouraged to carefully consider preanalytic variables with designing diagnostic tests systems and adapt collection, transport, and storage instructions accordingly.

3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (Garner JS, Hospital Infection Control Practices Advisory Committee. Guideline for isolation precautions in hospitals. *Infect Control Hosp Epidemiol*. 1996;17(1):53-80; and U.S. Department of Health and Human Services (BMBL), CDC, and NIH. *Biosafety in Microbiological and Biomedical Laboratories*, 4th Edition, 1999, available at: http://www.cdc.gov/od/ohs/biosfty/bmb14/bmb14toc.htm). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

4 Definitions

3′ poly(A) tail – a sequence of adenylyl residues at the 3′ end of eukaryotic mRNA; **NOTE:** Almost all mature eukaryotic mRNAs have 3′ poly(A) tails of 40 to 200 nucleotides, those of histones being a notable exception. The poly(A) tail is added enzymatically to the primary transcript, which is first cleaved 10 to 30 nucleotides past a highly conserved AAUAAA sequence. The poly(A) tail is then generated from ATP through the activity of polynucleotide adenylyltransferase. In practical terms, the poly(A) tail on mRNA has facilitated its ready isolation from total cellular RNA by affinity chromatography on oligo(dT) cellulose.

5′ cap – a structural feature present at the 5′ end of most eukaryotic (cellular or viral) mRNA molecules and also some virion mRNA molecules, but not of bacterial mRNA molecules; **NOTE:** It consists of a residue of 7-methylguanosine (m^7G cap) and a triphosphate bridge linking it 5′-5′ to the end of the polynucleotide chain. The cap structure is thought to protect the 5′ end of the mRNA from degradation by phosphatases or nucleases and to facilitate initiation of translation of mRNA by the eukaryotic (but not the bacterial) ribosome.

analyte – component represented in the name of a measurable quantity (ISO 17511)[1]; **NOTE 1:** In the type of quantity "mass of protein in 24-hour urine," "protein" is the analyte. In "amount of substance of glucose in plasma," "glucose" is the analyte. In both cases, the long phrase represents the measurand (ISO 17511)[1]; **NOTE 2:** In the type of quantity "catalytic concentration of lactate dehydrogenase isoenzyme 1 in plasma," "lactate dehydrogenase isoenzyme 1" is the analyte. The long first phrase designates the measurand (ISO 18153)[2]; **NOTE 3:** The analyte is the particular component of interest to the patient.

biomarker – a specific analyte (DNA, RNA, protein) found in a patient specimen which is useful for measuring the progress of disease or the effects of treatment.

degradation – the natural (hydrolysis), accidental (poor handling procedures), or induced (nuclease) destruction of a molecule into its component parts; especially as pertains to RNA integrity and stability.

disruption – complete breakage of cell walls and plasma membranes of solid tissues and cells, which is absolutely necessary to release all the DNA and RNA contained in the specimen and to release and inactivate endogenous nucleases; **NOTE 1:** Different specimen types (e.g., tumor tissues vs. PBMCs) require different methods to achieve complete disruption; **NOTE 2:** Incomplete disruption results in significantly reduced nucleic acid yields; **NOTE 3:** Compare with **homogenization** below.

DNA (deoxyribonucleic acid) – a type of nucleic acid; a polynucleotide having a specific sequence of deoxyribonucleotide units (dNTPs) and serving as the carrier of genetic information; **NOTE:** DNA is a double-stranded molecule held together by weak hydrogen bonds between base pairs of nucleotides. The four nucleotides in DNA contain the bases: adenine (A), guanine (G), cytosine (C), and thymine (T). Essentially, two forms of DNA can be distinguished: genomic DNA (gDNA) from the nucleus (nuclear DNA of the chromosomes), and mitochondrial DNA.

enrichment (nucleic acid or cellular) – to increase in content or abundance, e.g., to increase the abundance of one analyte within a complex mixture of nucleic acids or cellular components by the selective removal of other nonanalyte nucleic acids or cell components.

expression – the conversion of the genetic instructions present in a DNA sequence into a unit of biological function in a living cell; **NOTE:** Expression typically involves the process of transcription of a DNA sequence into an RNA sequence followed by translation of the RNA into protein; the RNA may be spliced before translation to remove introns.

genome – the complete genetic content of an organism.

genomics – the study of the genome, which includes genome mapping, gene sequencing, and gene function.

homogenization – the process by which high-molecular weight gDNA and other high-molecular-weight cellular components are sheared to create a homogenous lysate; it is necessary to reduce the viscosity of the cell lysates created by **disruption** (see above) prior to final isolation; **NOTE:** Incomplete homogenization results in inefficient binding of DNA and/or RNA and therefore significantly reduced yields during purification.

inhibitor – *in biochemistry*, any substance or agent that inhibits an enzymatic reaction, especially important for PCR-based clinical assays.

integrity (molecular, RNA) – a measure of functionality, typically of an RNA molecule, by assessing intactness (full-length), 3´ poly(A) and 5´ cap structures, as well as purity.

measurand – particular quantity subject to measurement (VIM93)[3]; **NOTE 1:** For example, vapor pressure of a given sample of water at 20 °C (VIM93)[3]; **NOTE 2:** The specification of a measurand may require statements about quantities such as time, temperature, and pressure (VIM93)[3]; **NOTE 3:** This term and definition encompass all quantities, while the commonly used term “analyte” refers to a tangible entity subject to measurement. For example, “substance” concentration is a quantity that may be related to a particular analyte; **NOTE 4:** The measurand describes what is causing the result of the measurement; and the analyte describes the particular component of interest to the patient.

oligo(dT) – oligodeoxynucleotide polymer of deoxythymidine, typically 20 to 80 DNA bases in length and covalently bound to matrix cellulose; **NOTE:** Used during mRNA isolation procedures; **NOTE:** See **3´ poly(A) tail** above.

Optimal Cutting Temperature (OCT) compound – a widely used embedding medium for tissues for histopathologic analysis.

proteome – the complete expression profile of proteins of an organism.

proteomics – the study of the proteome by the analysis of the protein structure and composition.

qualitative – a characterization applied to laboratory tests that detect and/or identify a particular analyte, constituent, or condition; **NOTE:** This term is applied to tests that detect whether a particular analyte, constituent, or condition is present or absent.

quantitative – a characterization applied to laboratory tests that give results expressing a numerical amount or level of an analyte in a specimen; **NOTE:** It is usually compared to an accredited recognized standard.

reproducibility (of results of measurements) – closeness of the agreement between the results of measurements of the same measurand carried out under changed conditions of measurement (VIM93).[3]

RNA (ribonucleic acid) – a single-stranded nucleic acid similar to DNA but having ribose sugar rather than deoxyribose sugar, and uracil rather than thymine; found in the nucleus and cytoplasm of cells, it plays an important role in protein synthesis and other chemical activities of the cell; **NOTE 1:** The structure of RNA is similar to that of DNA; **NOTE 2:** There are several classes of RNA molecules, including messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), and other small RNAs, each serving a different purpose; **NOTE 3:** A mixture containing all RNA entities is termed *total RNA*.

sample – one or more parts taken from a system, and intended to provide information on the system, often to serve as a basis for decision on the system or its production (ISO 15189).[4]

sample (patient) – a sample taken from the patient specimen and used to obtain information by means of a specific laboratory test.

specimen – biological material which is obtained in order to detect or to measure one or more quantities, such as amount or concentration (ISO/CD 18112-1)[5]; **NOTE:** Examples include whole blood, urine, stool, CSF, or solid tissues.

specimen (patient) – the discrete portion of a body fluid or tissue taken for examination, study, or analysis of one or more quantities or characteristics to determine the character of the whole.

target – the area of the nucleic acid to be detected or amplified for detection by a clinical test.

transcriptome – **(1)** the complete expression profile of mRNAs of an organism; **NOTE:** See **expression** above; **(2)** the full complement of activated gene mRNAs, or transcripts in a particular tissue at a particular time.

validation – confirmation through the provision of objective evidence that requirements for a specific intended use or application have been fulfilled (ISO 9000)[6]; **NOTE 1:** WHO defines validation as "the action (or process) of proving that a procedure, process, system, equipment, or method used works as expected and achieves the intended result" (WHO-BS/95.1793)[7]; **NOTE 2:** The components of validation

are quality control, proficiency testing, validation of employee competency, instrument calibration, and correlation with clinical findings.

whole blood – blood collected in an anticoagulant solution, with or without the addition of nutrients... {and not separated by sedimentation or centrifugation}(WHO-BS/95.1793).[7]

5 Specimen Collection

Appropriate specimen handling is critical to ensure specimen integrity and the accuracy of quantitative and qualitative nucleic acid detection. Specimens should be collected using all appropriate biosafety guidelines. Specimens must be collected, transported, and stored properly prior to testing. Inappropriate specimen handling can result in nucleic acid degradation, which can lead to erroneous quantitation of target from the patient.

5.1 Analyte/Specimen Matrix

Nucleic acids are isolated from a variety of sources. The following table is intended as a useful index for locating the sections in this document which pertain to applicable sample handling/preparation techniques for each nucleic acid/source pair.

Table 1. Analyte/Specimen Matrix*

Specimen or Sample Type	gDNA	vDNA	Bact DNA	mtDNA	Cell RNA	vRNA	Bact RNA
BAL		6.4.2 7.2.4 7.3.1 7.3.2	6.4.2 7.2.4 7.3.2			6.4.2 7.2.4 7.3.1 7.3.3	6.4.2 7.2.4 7.3.3
Bone Marrow	5.2.4 6.4.3.1 7.3.2				5.2.4 6.4.3.2 7.3.3		
Buccal Cells	6.4.4 7.3.2	6.4.4 7.3.1 7.3.2					
Buffy Coat	6.4.5 7.1 7.3.2 8.3	6.4.5 7.1 7.3.1 7.3.2 8.3		6.4.5 7.1 7.3.2 8.3	6.4.5 7.1 7.3.3 8.3		
CSF	6.4.6.1 7.2.1 7.2.3 7.2.4 7.3.2	6.4.6.1 7.2.1 7.2.3 7.2.4 7.3.1 7.3.2	6.4.6.1 7.2.1 7.2.3 7.2.4 7.3.2		6.4.6.2 7.2.1 7.2.3 7.2.4 7.3.3	6.4.6.2 7.2.1 7.2.3 7.2.4 7.3.1 7.3.3	6.4.6.2 7.2.1 7.2.3 7.2.4 7.3.3
Cultured Cells	6.4.7 7.2.4 7.3.2	6.4.7 7.2.4 7.3.1 7.3.2	6.4.7 7.2.4 7.3.2	6.4.7 7.2.4 7.3.2	6.4.7 7.2.4 7.3.3	6.4.7 7.2.4 7.3.1 7.3.3	6.4.7 7.2.4 7.3.3
CVS/Amniotic Fluid	5.2.6 7.3.2						
Fine Needle Aspirate	6.4.8 7.3.2	6.4.8 7.3.1 7.3.2	6.4.8 7.3.2	6.4.8 7.3.2	6.4.8 7.3.3	6.4.8 7.3.1 7.3.3	6.4.8 7.3.3
Frozen Tissue	5.2.5 6.4.9.1 7.1.4 7.3.2	5.2.5 6.4.9.1 7.1.4 7.3.1 7.3.2	5.2.5 6.4.9.1 7.1.4 7.3.2	5.2.5 6.4.9.1 7.1.4 7.3.2	5.2.5 6.4.9.2 7.1.4 7.3.3	5.2.5 6.4.9.2 7.1.4 7.3.1 7.3.3	5.2.5 6.4.9.2 7.1.4 7.3.3
Oral Fluid	6.4.10 7.3.2	6.4.10 7.3.1 7.3.2	6.4.10 7.3.2	6.4.10 7.3.2	6.4.10 7.3.3	6.4.10 7.3.1 7.3.3	6.4.10 7.3.3
Paraffin Embedded Tissue	6.4.11 7.1.4 7.3.2	6.4.11 7.1.4 7.3.1 7.3.2	6.4.11 7.1.4 7.3.2	6.4.11 7.1.4 7.3.2	6.4.11 7.1.4 7.3.3	6.4.11 7.1.4 7.3.1 7.3.3	6.4.11 7.1.4 7.3.3
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Serum/Plasma	6.4.1 7.2.2 7.2.3 7.3.2	6.4.1 7.2.2 7.2.3 7.3.1 7.3.2	6.4.1 7.2.2 7.2.3 7.3.2			6.4.1 7.2.2 7.2.3 7.3.1 7.3.3	6.4.1 7.2.2 7.2.3 7.3.3
Sputum			6.4.13 7.3.2				

Table 1. (Continued)

Specimen or Sample Type	gDNA	vDNA	Bact DNA	mtDNA	Cell RNA	vRNA	Bact RNA
Stool		6.4.14 7.2.5 7.3.1 7.3.2	6.4.14 7.2.5 7.3.2		6.4.14 7.2.5 7.3.3		6.4.14 7.2.5 7.3.3
Swabs		5.2.7 6.4.15 7.3.1 7.3.2	5.2.7 6.4.15 7.3.2				5.2.7 6.4.15 7.3.3
Tissue/Biopsy	5.2.5 6.4.9.1 7.1.4 7.2.6 7.3.2	5.2.5 6.4.9.1 7.1.4 7.2.6 7.3.1 7.3.2	5.2.5 6.4.9.1 7.1.4 7.2.6 7.3.2	5.2.5 6.4.9.1 7.1.4 7.2.6 7.3.2	5.2.5 6.4.9.2 7.1.4 7.2.6 7.3.3	5.2.5 6.4.9.2 7.1.4 7.2.6 7.3.1 7.3.3	5.2.5 6.4.9.2 7.1.4 7.2.6 7.3.3
Urine	6.4.16 7.2.1 7.2.4 7.3.2		6.4.16 7.2.1 7.2.4 7.3.2		6.4.16 7.2.1 7.2.4 7.3.3		6.4.16 7.2.1 7.2.4 7.3.3
Whole Blood	5.2.4 6.4.1 6.4.1.1 7.3.2 8.3	5.2.4 6.4.1 7.3.1 7.3.2 8.3	5.2.4 6.4.1 7.3.2 8.3	5.2.4 6.4.1 6.4.1.1 7.3.2 8.3	5.2.4 6.4.1 7.3.3 8.3	5.2.4 6.4.1 7.3.1 7.3.3 8.3	5.2.4 6.4.1 7.3.3 8.3

*Key of Abbreviations:

Bact DNA	bacterial DNA
Bact RNA	bacterial RNA
BAL	bronchoalveolar lavage
Cell RNA	cellular RNA (transfer, messenger, and ribosomal RNA)
CSF	cerebrospinal fluid
CVS	chorionic villus sampling
gDNA	human genomic DNA
mtDNA	mitochondrial DNA
vDNA	viral DNA
vRNA	viral RNA

NOTE: Sections 6.2 and 8.1 of this document apply to DNA testing for all specimen sources. Sections 6.3 and 8.2 apply to RNA testing for all specimen sources.

5.2 Specimen Labeling and Collection

Specimens should be collected, labeled, handled, and stored in a manner that respects patient privacy with respect to their medical records and medical data in accordance with HIPAA regulations available at http://www.hhs.gov/ocr/hipaa/finalreg.html. The intent of the HIPAA regulations, however, is not to prevent appropriate sharing of information sufficient to treat the patient among healthcare professionals directly involved in patient treatment. Thus, due respect to patient privacy should be observed at all times.

5.2.1 Specimen Identification

The patient and the patient's specimen must be positively identified at the time of collection. Specimens should be labeled and handled in a manner that respects patient privacy in accordance with HIPAA regulations, yet provides sufficient information for medical personnel to appropriately perform the testing and treat the patient. These privacy regulations are available at the following website: http://www.hhs.gov/ocr/hipaa/finalreg.html.

Specimens for molecular testing must be identified with a firmly attached label bearing at least the following:

- an identification number;
- the date of collection;
- the time of collection;
- name of the person collecting the specimen (optional); and
- specimen source (i.e., type of tissue from which the specimen was taken).

5.2.2 Information for Request Form

The following information should be included:

- a unique identification number;
- an accessioning number;
- patient name;
- date of birth;
- date of collection;
- gender (if needed for genetic analysis);
- race/ethnicity (if applicable);
- specimen type (blood, amniotic fluid, etc.);
- relevant clinical and laboratory information;
- the doctor's name;
- the department or location where the specimen was collected;
- billing information (if applicable); and
- other information as needed.

NOTE: In some cases, additional information may be necessary. For example, in the case of genetic tests, the reason for requesting the test may be necessary because the laboratory director may need to determine whether or not the test is appropriate given the situation and age of the patient. Also, for some genetic tests, pedigree information may be needed or required (linkage analysis, for example).

5.2.3 Specimen Collection

Gloves should be worn when handling human tissue or body fluids. Gloves can prevent transmission of blood-borne pathogen infection from a specimen, and contamination of the sample by exfoliated cells from persons handling the sample. All applicable safety precautions regarding handling body fluids should be followed. (See the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections* and U.S. Department of Health and Human Services (BMBL), CDC, and NIH. Biosafety in Microbiological and Biomedical Laboratories, 4th Edition, 1999 (http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm).

Specific testing procedures (or methods) may require additional precautions and collection instructions (e.g., collecting cervical specimens for HPV testing before acetic acid visualization). Identifying all potential interfering substances that may be commonly used in specific clinical settings for collection of specific specimen types is beyond the scope of this document. Laboratorians performing different testing methods should consider potential sources of interference and contamination, and ensure that clinicians collecting specimens are appropriately advised and trained with specimen collection requirements that may be unique for specific testing methods or test systems. Additional precautions and specialized collection materials may also be recommended or required for specific test systems.[8,9]

Specimens received in a clinical laboratory should be entered in a laboratory (or hospital) information system as soon as possible.

Every attempt should be made to process submitted specimens. However, the following specimens are considered unacceptable: hemolyzed blood, frozen blood,[10] and improperly labeled specimens and samples. Ordinarily, there is no exception for the rejection of mislabeled or unlabeled specimens/samples. A rare exception, however, may be considered in special circumstances and when authorized by a laboratory director or designee at the request of the treating physician, if testing is absolutely required and the specimen can be salvaged for appropriate laboratory testing. All nonroutine requests must be documented. The sample acceptability criteria must be set for each assay and should be part of the laboratory's standard operating procedure manual for that assay.

5.2.4 Anticoagulant Used in Blood and Bone Marrow Aspirate (BMA) Collection

Specimens are collected in appropriate anticoagulant- or additive-containing tubes. The tube additive chosen depends upon the measurand (e.g., gDNA, vRNA, intracellular RNA), the tests to be performed, and the volume of the sample required. Several studies have shown that heparin anticoagulant and heme are potent inhibitors of PCR[11-13]; therefore, ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests requiring plasma or bone marrow aspirate (BMA) samples. If the measurand is intracellular RNA, it is recommended that blood or bone marrow be collected in a device containing an RNA stabilization additive, or put in an RNA stabilizing solution as soon after collection as possible.

5.2.5 Tissue Specimen Collection Requirements

Tissue specimens are used when blood or buccal cells are not available (e.g., a patient is deceased), when tissue and blood or buccal cells have a different genotype from the tissue to be tested (e.g., for somatic mutations in neoplastic diseases, or for mosaicism), or when tissue is the only source of nucleic acids from potential infectious agents.

An optimal amount of tissue is usually 1 to 2 g, but this optimal amount depends on the nature of tissue, since the amount of DNA and RNA per weight varies greatly from tissue to tissue. Highly cellular tissue such as bone marrow, lymph node, and spleen are suitable sources for genomic DNA and may require less tissue. It should be kept in mind, however, that lymph nodes downstream from a primary tumor may harbor a small number of tumor cells that could go undetected by light microscopic examination of a single tissue section. Hypocellular specimens such as muscle, fibrous, and adipose tissues are not an optimal source of genomic DNA, and may require more than the 1 to 2 g. In general, however, any amount of tissue weighing more than 10 mg should yield >10 µg of RNA or DNA if there is not extensive fatty infiltration of the source tissue. Because of highly variable amounts and types of proteins present in source tissues, nucleic acid isolation protocols are tissue-specific. Follow manufacturers' recommendations for DNA and RNA isolation procedures for particular source tissues.

Large tissue specimens (>1 to 2 g) and biopsy tissue are procured by a clinician or surgeon for pathologic diagnosis by light microscopy, electron microscopy, and/or immunofluorescence microscopy. If DNA or RNA is to be extracted from a biopsy or large tissue specimen, care should be taken to maintain the hydration of the tissue. The tissue should be wrapped in sterile gauze or paper soaked with sterile normal saline.

The pathologist typically takes representative tissue sections from large tissue specimens or biopsy tissue for fixation, staining microscopic examination, and pathologic diagnosis. The pathologist can also take representative tissue samples to extract DNA or RNA for molecular analysis. The selected tissue sample may either be processed for the production of cell lines or homogenized for the recovery of biomolecules (i.e., DNA, RNA, and proteins). The pathologist can usually identify lesional tissue for testing and

nonlesional tissue that may serve as a control. Control tissues are sometimes essential for some molecular assays, such as loss of heterozygosity or microsatellite instability assays.

The stability of DNA and RNA in tissue specimens varies with tissue type. In general, storage of tissue at 22 to 25 ^{o}C is not recommended. For optimum results, the tissue should be snap frozen in liquid nitrogen or placed in a suitable nucleic acid preservative. If this is not possible, the tissue specimen should be placed immediately on wet ice and subsequently transported on ice for better preservation of nucleic acids, particularly RNA. Small specimens may be wrapped in gauze soaked with normal saline to prevent the specimen from drying. The tissue specimen is typically submitted to a pathology department of the hospital and examined by a pathologist. Tissue samples to be submitted for analysis of DNA or RNA should be placed in a suitable stabilizing solution as soon as possible to prevent nucleic acid degradation. This is particularly critical for RNA transcripts, some of which have half-lives of seconds or minutes. It should be noted, however, that most available stabilizing additives are not adequate as fixatives for traditional histological or immunohistological analysis.

It should be noted that, because of the anesthesia required and the lack of standardized nucleic acid stabilization technologies, surgical procedures in particular may cause tissue anoxia, which in turn produces changes in expression levels of many genes. Prolonged tissue hypoxia decreases pH in tissue locally, resulting in a decrease in the yield of nucleic acids.[14]

5.2.6 Prenatal Specimens

Prenatal specimens include chorionic villus sampling (CVS) specimens, cultured CVS cells, amniotic fluid, cultured cells from amniotic fluid, and any other cellular specimens derived from the fetus prior to delivery. These specimens are used to predict the genotype and phenotype of the fetus prior to delivery, facilitating medical/surgical intervention to the fetus if necessary. Fetal specimens must be labeled with the mother's full name and type of fetal specimen. Maternal blood should accompany the specimen in order to carry out an analysis to exclude significant maternal cell/DNA contamination. A backup culture must be maintained until testing is complete and an additional sample is no longer necessary. Amniotic fluid can be processed without culture after 15 weeks of gestation. A standard amount of a direct amniotic fluid specimen is at least 10 mL.

CVS specimens must be cleaned to remove maternal tissue, especially endometrial decidual tissue, prior to shipping or processing. A standard amount of a CVS specimen is at least 15 mg after removal of maternal tissue if any. A CVS specimen should be shipped in sterile tissue culture medium or saline buffer at ambient temperature. Cultured cells from either amniotic fluid or CVS specimen should be shipped in two plastic culture flasks with greater than 75% confluent cells filled completely with culture medium.

A CVS specimen should be examined under a dissecting microscope to assess contamination of maternal tissue. If it appears free of maternal contamination, it should be processed on the same day of arrival. If the specimen does not appear free of maternal contamination, tissue that appears maternal should be removed manually as much as possible. If the specimen cannot be processed on the same day of arrival, it should be stored at 2 to 8 °C and the DNA extracted the next day.

DNA should be extracted from an amniotic fluid specimen on the same day of arrival or, if it cannot be processed on the same day, the specimen should be stored at 2 to 8 °C and the DNA extracted the next day.

A cultured CVS or cultured amniotic fluid cell sample should be examined for cellular confluency by an inverted phase microscope as soon as possible. If it cannot be processed within two hours of arrival, it should be stored at 2 to 8 °C. In general, at least 75% confluency is required for testing. If cells are less

than 75% confluent, a laboratory director may be consulted to continue culture of the cells. If a sample is acceptable for testing, it should be processed on the same day as its arrival at the laboratory.

5.2.7 Cervical and Urethral Swabs

Male urethral samples are collected with polyester-tipped swabs, either stainless steel shaft or flexible, plastic shaft. Female endocervical and vaginal samples are collected with either rayon- or polyester-tipped swabs and placed into the appropriate transport media as specified by the assay manufacturer. Samples collected for HPV are collected using the swabs recommended by the manufacturer of the assay and placed into the transport system specified by the manufacturer of the assay.

6 Specimen Transport and Storage

While these recommendations are general guidelines to follow when transporting specimens for molecular testing, laboratories should follow manufacturers' directions for collection and transport of samples for use with molecular diagnostic test kits. Laboratories should also follow state, local, and federal regulations. The laboratory must provide instructions for proper specimen handling and transport conditions to those who collect and transport specimens. Special handling requirements should also be provided to couriers responsible for the transport of specimens. It is equally important to note the following for every specimen: date and time of collection, date shipped, date received by the laboratory, and approximate temperature of specimen when received by the laboratory. In addition, laboratories may rely upon their own experiences when dealing with the different specimen types.

One of the major issues in nucleic acid testing is the proper storage conditions for blood and other specimen types to obtain reliable results. During transport and storage, the specimen must not be exposed to conditions that might result in degradation of target nucleic acids. The storage conditions vary depending on specimen type, analyte (RNA or DNA), and/or microorganism being tested. Proper transport and storage conditions must be determined by the assay manufacturer or, in the case of "home-brewed" tests, by the laboratory. RNA is highly susceptible to degradation and can be more difficult to recover than DNA. Furthermore, certain genes in extracted tissue or blood samples can be induced by the sample collection process, which causes an artifactual increase in corresponding mRNAs. This should be taken into consideration when performing quantitative gene expression analysis of tissues and blood.

6.1 General Recommendations for Transport

6.1.1 Regulations

Regulations regarding packaging and shipment of clinical/diagnostic samples will differ from country to country. These regulations are beyond the scope of this document, but can be found using the following links:

- http://www.cdc.gov/od/ohs/biosfty/biosfty.htm
- http://www.cdc.gov/od/ohs/pdffiles/DOTHazMat8-14-02.pdf
- http://www.who.int/csr/resources/publications/WHO_CDS_CSR_LYO_2004_9/en/

6.1.2 Irradiation

Due to recent events in the United States, some shipments may eventually be sterilized by gamma irradiation or particle beam emission equipment. Very little research has been done to examine the effect of irradiation on clinical specimens, especially those that will be used for molecular testing. Of the research that has been done, one study looked at the effect of irradiation on protein or drug analytes in whole blood and in dried blood spots.[15] A separate study determined the effect of irradiation on protein analytes and DNA in dried blood spots used for proficiency testing and quality control materials for

newborn screening programs.[16] It is not possible at this time to draw conclusions for all specimen types and analytes. Much more research is required to address this issue.

6.2 General Recommendations for Storage of Purified DNA

It is generally recommended that purified DNA samples be kept below the freezing point of water for long-term storage to minimize degradative activity of DNases. Store purified DNA samples in a tightly capped, hydrophobic, plastic tube, preferably with a rubber gasket to prevent evaporation. It should be noted that some tubes are more suitable than others for storage of DNA. Polypropylene tubes are associated with DNA adsorption, especially at high ionic strength. Polyethylene tubes bind DNA even more strongly than polypropylene. Polyallomer tubes and some specially designed polypropylene tubes have been shown to be appropriate specifically for storing DNA.[17]

Purified DNA can be stored safely in TE (Tris-EDTA) buffer at room temperature for 26 weeks, at 2 to 8 °C for at least one year if contaminating DNases are absent (consensus opinion, CAP Ed. BD.), and for up to seven years at -20 °C[18] and at least seven years at -70 °C or lower.[10,19] Samples of questionable purity should be stored at or below -20 °C to ensure DNA integrity. Freezers used to store purified DNA should not be of the "frost-free" variety, as these freezers continually cycle temperatures, causing deterioration of the nucleic acids by shearing.

6.3 General Recommendations for RNA Studies

Both degradation of RNA and gene induction can occur following blood specimen collection, causing changes in the *in vivo* gene expression profile.[20] The same phenomena can also occur within minutes in tissue or body fluid samples. It is therefore recommended that wherever possible, blood samples should be drawn directly into a tube containing an RNA stabilization additive. Tissue samples should be placed immediately into a vial containing an RNA stabilizing agent or flash-frozen in liquid nitrogen. Frozen specimens should be transported on dry ice, and not be thawed prior to RNA extraction. Regardless of how long storage will be, -70 °C or lower is the recommended temperature, since ribonucleases (RNases) continue to degrade RNA even at -20 °C.

The influence of different anticoagulants and preamplification storage conditions on the stability of various viral RNAs has been reported earlier (see Section 5.2.4).

6.4 Recommendations for Collection, Transport, and Storage of Specific Specimen Types

The reliability and accuracy of molecular test results depend upon a variety of specimen collection, transport, and storage factors. These include: collection procedures, the nature and location of target nucleic acid (whether DNA or RNA, host genomic or pathogen nucleic acids), and the type of testing to be done.

If pathogen nucleic acids are targeted, the life cycle of the pathogen, and the type of cells in which they replicate would also be important in determining specimen type, method of collection, and processing. The following recommendations and discussions are general; therefore, specific analytical and clinical variables as well as the type of testing that will be performed should be taken into consideration. Furthermore, although manufacturers of molecular diagnostic assay kits may perform studies to support recommended storage and transport conditions, conclusions from these studies may not be generalizable and may be limited by testing conditions. These recommendations are consistent with available evidence (which may be limited) and experience within different laboratory settings, and should be used as general guidelines.

6.4.1 Whole Blood, Serum, and Plasma

Although EDTA is the preferred anticoagulant for the collection of whole blood and the production of plasma for molecular test methods, it can interfere with downstream assays. The laboratory should follow the directions in the assay manufacturer's product insert for specimen collection, transport, and storage. When EDTA is used, whole blood can be collected in tubes with or without a gel separator. For RNA targets such as HIV or HCV, whole blood samples should be centrifuged and, in the case of a nongel separator tube, the plasma removed to a secondary tube within four hours of phlebotomy. Plasma separated in a gel separator tube may be transported to the laboratory *in situ.* Plasma samples are stable for up to five days at 2 to 8 °C and longer if frozen at -20 °C or -70 °C or lower. The laboratory should validate the effects on analytical results of *in situ* freezing of the plasma sample in gel separator tubes and freeze-thaw cycles of plasma stored in secondary tubes.

Blood scheduled for DNA analysis can be stored at room temperature for up to 24 hours or at 2 to 8 °C for up to 72 hours prior to DNA extraction. Blood scheduled for cellular RNA analysis should be collected in a tube containing an RNA stabilizing additive. Collection and storage of unstabilized whole blood is not recommended for gene transcription analysis, because artifactual gene induction and RNA degradation occurs in blood stored *ex vivo*.

Serum should be shipped frozen on dry ice for either DNA or RNA studies.[21] Plasma should be shipped at 2 to 8 ºC and stored at -20 ºC.[22]

For RNA studies, extraction should begin within four hours, or the specimen should be frozen.[22] For long-term storage, serum, plasma, or blood cell nuclei can be prepared and stored at -20 °C or -70 °C or lower.

Do not store plasma samples in a "frost-free" freezer. The temperature is cycled several times per day in this variety of freezer, causing degradation of nucleic acid targets. HIV viral load measurements vary with different handling conditions. It is important to standardize specimen collection and processing for these types of tests.

6.4.1.1 Dried Blood Spots

Dried blood spots (DBS) are suitable for DNA analysis and are not recommended for studies requiring intact RNA. Once air-dried, DBS should not be placed in hermetically sealed bags, since retained moisture encourages growth of microorganisms. If placed in plastic bags or containers, sufficient desiccant to minimize moisture exposure should be placed in the container. Either an indicator desiccant or a humidity card may be included to indicate whether the specimen has experienced excessive moisture.

To prevent cross-contamination, multiple DBS specimens should be separated by glassine paper (laboratory weighing paper), a paper coverslip, or by positioning them in such a way that they are not in contact with each other. DBS may be transported at room temperature. (For additional information, see the most current edition of CLSI/NCCLS document LA4—*Blood Collection on Filter Paper for Newborn Screening Programs.)* Specimens collected on filter papers have been found to be suitable for PCR analysis for at least 19 months.[23]

6.4.2 Bronchoalveolar Lavage (BAL)

Bronchoalveolar lavage (BAL) specimens should be collected and aliquoted according to the manufacturer's instructions or protocol for the assay to be performed. Specimens should be transported and tested within 24 hours of collection.[22] Those samples, which cannot be assayed within 24 hours of collection, should be refrigerated for up to 72 hours at 2 to 8 °C or frozen at -70 °C or lower for future testing. Specimens for mycobacteria should be decontaminated and digested prior to freezing or long-term storage.[24]

6.4.3 Bone Marrow Aspirate

6.4.3.1 DNA

Bone marrow should be aspirated into a syringe containing an EDTA anticoagulant. Heparin is not recommended as an anticoagulant for molecular testing. Laboratory personnel responsible for specimen receipt and processing should be notified as soon as possible that a bone marrow specimen has arrived. For DNA extraction, bone marrow specimens can be stored temporarily at 2 to 8 °C prior to processing. Processing to the cell lysis step of the nucleic acid extraction procedure should occur within 72 hours of receipt. If long-term storage is required, bone marrow specimens can be stored at -20 °C for several months if erythrocytes are first eliminated.

Although varying success with extraction of intact DNA from frozen whole EDTA anticoagulated blood has been reported, freezing of whole blood prior to DNA extraction is not recommended. This is also true for samples containing blood, including bone marrow aspirates and bone marrow core biopsies. These specimen types should not be frozen unless processed to remove red blood cells. When blood or bone marrow specimens are frozen and thawed, red cell lysis occurs, causing the release of heme, a known inhibitor of PCR.[25]

6.4.3.2 RNA

Laboratory personnel responsible for specimen receipt and processing should be notified as soon as possible that a bone marrow specimen has arrived.

Bone marrow should be aspirated into a syringe containing an EDTA anticoagulant. Heparin is not recommended as an anticoagulant for molecular testing. For RNA studies, bone marrow aspirate should be put into an RNA stabilization solution as soon as possible. If this is not possible, the BMA should be placed on wet ice immediately and transported to the laboratory. RNA extraction should begin within one to four hours if the specimen is not stabilized and cannot be frozen.[22] Samples should not be frozen unless erythrocytes are first eliminated.

6.4.4 Buccal Cells

Both DNA and RNA can be isolated from buccal cells. Mouthwash specimens are also commonly used as a source of buccal cells. An RNA stabilizing agent should be used to preserve RNA in buccal cell and mouthwash specimens. Buccal cells scheduled for DNA testing can be collected on appropriate swabs, dried, and transported at ambient temperature. Mouthwash specimens scheduled for DNA testing may be transported at room temperature,[26-28] and they are stable at room temperature for approximately one week. Saliva scheduled for RNA testing must be collected and transported in a suitable RNA stabilizing agent.

6.4.5 Buffy Coat

For DNA testing, if DNA cannot be extracted from blood within three days of collection, the buffy coat may be isolated and stored at -70 ºC or lower prior to DNA testing.[29] Buffy coat specimens that are being used for immortalization by Epstein-Barr virus should be transported frozen on dry ice.[30,31] RNA should be isolated from buffy coat within one to four hours of specimen collection. Alternatively, cells can be placed in an RNA stabilization solution and stored at room temperature until isolation. In patients with eosinophilia, high levels of endogenous ribonuclease can prove problematic with buffy coat samples.[32,33]

6.4.6 Cerebrospinal Fluid (CSF)

6.4.6.1 DNA

Cerebrospinal fluid should be transported at 2 to 8 ºC for DNA studies. If specimens cannot be processed immediately, CSF specimens tested for DNA viruses (e.g., HSV, CMV, EBV, and VZV) should be placed at -20 or -70 °C or lower.

6.4.6.2 RNA

CSF specimens for RNA studies (including for RNA viruses, e.g., enterovirus) must be chilled immediately on wet ice and the RNA extracted within one to four hours of collection.[22] If this is not possible, specimens may be immediately frozen after contaminating red blood cells are removed. Frozen CSF specimens should be shipped to the laboratory on dry ice.

6.4.7 Cultured Cells

Continuous incubation at 37 ^{o}C is recommended until cells are harvested for nucleic acid (DNA or RNA) extraction. If this is not possible, some cultured cells, such as amniocytes and lymphoblasts, can be transported in a sufficient amount of culture media at room temperature.[21,31] Other cell types, such as human chondrocyte suspensions, may require refrigeration during transport and storage.[34] Follow guidelines for stabilization of DNA or RNA, as stated in Sections 6.2 and 6.3.

6.4.8 Fine Needle Aspirate (FNA)

For DNA extraction, follow the guidelines for BMA in Section 6.4.3.1 above. For RNA studies, FNA should be chilled immediately or placed in an RNA stabilization solution. However, if a specimen contains erythrocytes, it should be processed to remove the erythrocytes before adding RNA stabilization solution. RNA should be extracted from chilled specimens within two to four hours of specimen collection. If this is not possible, FNA samples can be frozen at -70 ^{o}C or lower. If a specimen contains erythrocytes, it should be processed to remove them before freezing. Frozen samples are stable for two to four weeks. Follow the manufacturer's recommendations for stability of specimens placed in RNA stabilization solution.

6.4.9 Tissue

6.4.9.1 DNA

For DNA extraction, tissue should be chilled immediately and transported on wet ice to the laboratory, where it should be stored at 2 to 8 ^{o}C for no longer than 24 hours prior to processing. Alternatively, tissue may be snap frozen at the collection site. Samples scheduled for *in situ* molecular analysis, such as fluorescent *in situ* hybridization (FISH), should be placed in optimal cutting temperature (OCT) medium and kept frozen until further processing. In general, DNA is stable in tissue for up to 24 hours at 2 to 8 ^{o}C, for at least two weeks at -20 ^{o}C, and for at least two years at -70 ^{o}C or lower.

If DNA is to be extracted from fresh tissue immediately upon arrival to the laboratory, notify responsible personnel assigned to extract nucleic acids when a fresh tissue specimen is expected to arrive, so preparations can be made for its timely processing. Solid tissue, particularly tumor tissue, is a rich source of endogenous nucleases. If it cannot be processed immediately, it should be stored at -70 °C or lower until processing. A fresh tissue specimen stored for subsequent DNA extraction should be frozen as promptly as possible. Bloody tissue should be washed by sterile saline before freezing. For optimal results, tissue is snap frozen in liquid nitrogen or in -70 °C or lower isopentene bath. For long-term

storage, -70 °C or lower is the recommended temperature. A tissue specimen should never be stored in a frost-free freezer, which makes freeze-thaw cycles, resulting in degradation of nucleic acids.

A fresh tissue specimen in Roswell Park Memorial Institute (RPMI) culture medium should be processed as quickly as possible. If a tissue specimen requires only nucleic acid analysis, it should be transported on wet ice to the laboratory. A fresh tissue specimen that also requires cytogenetic or flow cytometric analysis should be transported at ambient temperature to maintain cell viability.

6.4.9.2 RNA

If RNA is to be extracted from the tissue sample, the samples must be either snap frozen prior to storage at -70 ^{o}C or lower, placed in a stabilizing solution, or processed for RNA extraction within one hour of collection. RNA is stable in tissue after snap freezing for at least two years at -70 ^{o}C or lower, the preferred storage temperature for specimens containing RNA. Frozen samples scheduled for *in situ* molecular analysis (such as FISH) should be placed in OCT medium and kept frozen until further processing.

Tissue specimens that have been flash frozen in liquid nitrogen should be transported on dry ice and stored at -70 ^{o}C or lower until RNA extraction. Frozen specimens should not be thawed prior to extraction, but homogenized directly in guanidinium isothiocyanate buffer, or other suitable extraction media. If flash freezing or immediate stabilization is not possible, RNA must be isolated from the specimen within four, and preferably within one hour after collection. Processed, purified RNA is best stored as a precipitate in ethanol at -70 ^{o}C or lower.

A frozen tissue specimen in OCT medium should be transported on dry ice and should be stored in a -70 °C or lower freezer within two hours of receipt. If it is delivered to a laboratory frozen on wet ice, it must be placed immediately in a -70 °C or lower freezer to prevent thawing. If it has thawed during delivery, it should be processed as quickly as possible, or must be placed immediately in a -70 °C or lower freezer if immediate processing is not possible.

Since RNA is subject to degradation by RNases, store tissue specimens in sterile, hydrophobic, plastic tubes that have not been handled with ungloved hands.[35,36] Freezers used to store original specimens or purified nucleic acids should not be of the "frost-free" variety, as these freezers continually cycle temperatures, causing deterioration of the nucleic acids by shearing.

Solid tissue, particularly tumor tissue, is a rich source of endogenous nucleases. Therefore, a fresh tissue specimen stored for subsequent RNA extraction should be snap frozen as soon as possible in liquid nitrogen or in -70 °C or lower isopentene bath. Alternatively, samples scheduled for RNA extraction but not for histological examination can be placed in an RNA stabilizing reagent. Bloody tissue may be washed by sterile saline before being snap frozen.

Regardless of how long storage will be, -70 °C or lower is the recommended temperature, since ribonucleases (RNases) continue to degrade RNA even at -20 °C.

6.4.10 Oral Fluid

In general, the storage and transport for oral fluid scheduled for DNA or RNA testing is similar to that of a buccal specimen; except if the specimen is scheduled for RNA testing, it should be placed in a transport medium or stabilizing solution that stabilizes the RNA or refrigerated at 2 to 8 ºC. Oral fluid specimens should be processed within 24 hours of collection.[22]

6.4.11 Formalin-Fixed Paraffin Embedded Tissue (FFPET)

An FFPET sample, in a tissue block can be stored at room temperature indefinitely for future DNA analysis. It is possible to extract high-molecular-weight DNA from FFPET by critical point drying.[37] Mercury-based fixatives (e.g., B5 fixative) should be avoided.

While FFPET samples are not recommended for RNA studies, it may be possible to extract RNA from an FFPET sample either in a tissue block or on an unstained slide.[38-40]

An FFPET sample is normally used only if no other samples are available, because it is not the optimal specimen type.

6.4.12 Semen

Semen should be immediately chilled, transported on the day of collection, and maintained at 2 to 8 ºC until DNA extraction. Semen DNA analysis can be performed on dried semen, as well as semen on a fixed slide for cytological analysis and *in situ* hybridization techniques.[22]

6.4.13 Sputum

Sputum for DNA analysis should be collected in a sterile container and transported to the laboratory at room temperature. Specimens delayed longer than 30 minutes before transport should be refrigerated,[41] and if transport time is anticipated to be longer than 30 minutes, samples should be transported to the laboratory at 4 to 8 °C.

Upon receipt in the laboratory, specimens not tested immediately should be refrigerated. While samples collected for *Mycobacterium* testing by molecular methods may be stable for longer periods, samples should be processed promptly when the specimen is used for both culture and molecular test methods to optimize turnaround times (i.e., ability to report *M. tuberculosis* complex within 14 to 21 days from receipt of specimens). If longer storage times are needed, the specimen can be stored for at least one year at -70 ºC or lower.[42] As with all specimen types, the assay manufacturer's recommendations for storage and transport should be followed when using commercial extraction kits.

6.4.14 Stool

Stool should be shipped following the recommendations for the test being requested. Some methods may require a preserved specimen transport container[43]; others may require a stool that has been collected in a screw-cap container with no preservative and transported under refrigeration at 2 to 8 ºC.[44]

6.4.15 Cervical and Urethral Swabs

Generally, an adequate endocervical specimen contains metaplastic cells, and/or columnar or cuboidal endocervical cells, and quality will depend on appropriate collection methods. Swabs, brushes, or other collection devices (e.g., brooms) should be placed in a transport medium and transported, or transported dry in a sealed tube as recommended by the assay manufacturer or by the laboratory performing the assay. Some swab types have been reported to cause interference with some molecular assays. Depending on the downstream testing, DNA may be stable at 2 to 8 ºC for up to ten days.[22,31,45] Upon arrival at the laboratory, the swabs are resuspended, according to the manufacturer's instructions, in the transport medium. For further testing, a required amount of the transport fluid may be either stored at -70 ºC or lower or immediately centrifuged, and the pellet processed for DNA or RNA according to the assay manufacturer's recommendations. Frozen samples are thawed later for testing, centrifuged, and then processed in the same manner as fresh samples. Transport media provided for molecular testing may contain detergents that will lyse cells that are important for cytologic assessment. For test methods done

on crude specimen lysates (no purification, separation, isolation, or concentration of nucleic acids), care should be used to ensure optimal conditions for target recovery. For example, if nucleic acid targets are microbial, the proportion of host cellular DNA, presence/absence of other organisms, and amount of secretions or discharge may affect success of downstream assays.

6.4.16 Urine

Volume of urine, time since last urination, presence of inflammation, and other factors may affect recovery of target nucleic acids. Ambient temperature storage of fresh unprocessed urine should be minimized, since the low pH and high urea content rapidly denature DNA, especially at 25 °C and above.[46] Further processing is carried out following the manufacturer's directions for the type of assay to be performed. Once processed, samples may be stored at 2 to 8 °C. For RNA studies, follow instructions for specimen collection, transport, and storage recommended by the assay manufacturer or the laboratory performing the assay.

7 Sample Preparation

A variety of methods are used to prepare specimens for nucleic acid analysis.

7.1 Cell Enrichment/Selection

To obtain greater specificity and sensitivity in molecular assays, laboratories are increasingly analyzing homogeneous cell samples instead of larger tissues that may contain mixed cell populations. Laser capture microdissection, FACS sorting, and magnetic bead capture are among the methods developed to obtain cells of the same type from tissue or from blood. These methodologies are commonly used for the detection of circulating tumor cells as in the monitoring of minimal residual disease (MRD), or for the detection of fetal cells in maternal blood. Tumor cells can be singled out from a mixture of cells, either in tissue or circulating by using specific markers that identify the cells. Surface markers such as CD markers can be utilized to select for (positive selection) or deplete (negative selection) a certain population of cells.

Enrichment (isolation) of individual cells or single cell populations can be achieved by several methodologies:

- Isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation;
- Enrichment of cells based on density (elutriation);
- Enrichment using antibodies (FACS, magnetic bead capture)

  - To surface markers (CD antigens)

  - To tumor cell markers (epithelial antigens such as CK); and

- Laser capture microdissection - LCM (described in Section 7.1.4).

7.1.1 Isolation of PBMCs by Density-Gradient Centrifugation

Isolation of mononuclear cells from peripheral blood is accomplished by centrifugation of anticoagulated whole blood through a density gradient medium such as polymerized sucrose.

Whole blood can be layered on a commercially available density gradient medium and centrifuged according to the gradient medium manufacturer's instructions for use. Alternatively, samples can be

collected by phlebotomy directly into blood collection tubes, which contain density gradient media and which are designed for this purpose. Blood samples and density gradient medium should be brought to ambient temperature prior to layering and centrifugation for best results. If blood collection tubes designed for mononuclear cell separation are used, follow the tube manufacturer's instructions for use.

7.1.2 Enrichment of Cells Based on Density (Elutriation)

In centrifugal elutriation, cells are separated according to their sedimentation rate in a gravitational field where the liquid containing the cells is made to flow against the gravitational force. The liquid can be culture medium with a low density, so the sedimentation rate of the cells is proportional to their size. The elutriator rotor has two chambers connected to a channel system. When cells are loaded in the first chamber, the counterflow and rotating speed are adjusted so that the cells stay in suspension (i.e., the counterflow and the sedimentation rate must be in balance). The biggest cells accumulate at the bottom of the chamber and the smallest cells towards the top of the chamber near the center of the rotor. Different flow rates are used to remove (elute) the different fractions; higher flow rates are able to elute larger cells. A gradual increase in flow rate elutes each fraction sequentially. Counterflow elutriation (CCE) is a nonimmune, nondestructive lymphocyte depletion method, separating cells by sedimentation velocity. Cells removed by CCE remain viable.

7.1.3 Enrichment of Cells Using Antibodies

7.1.3.1 Fluorescent Antibody Cell Sorting (FACS)

Sorting can be defined as the physical separation of a cell or particle of interest from a heterogeneous population. Cells are labeled with different fluorescent markers and then separated or sorted based on their fluorescence profile.

7.1.3.2 Adsorption of Cells to Antibody-Coated Magnetic Beads

Antibody-coated magnetic beads are available for positive or negative selection of specific cell subsets from whole blood or buffy coat. Cells are separated from whole blood or buffy coat by incubating the blood with modified magnetic beads and passing the mixture through a separation column that is placed in a magnetic field, or by placing the tube with the cell bead mixture in a magnetic field. For positive cell selection, the cells of interest are retained in the column or tube, while unwanted cells pass through or are washed out. For negative cell selection, unwanted cells are retained on the column or in the tube, and the cells of interest are allowed to pass through or are washed out. After removal of the column tube from the magnetic field, the magnetically retained cells are eluted. The positively selected cells can be detached from the beads by one of two methods:

- *A universal detachment method specifically designed for positive isolation of any cell type*. In this system, the antibodies are attached to the surface via a DNA linker. This linker region provides an enzymatically cleavable site to remove the beads from the cells after isolation.

- *A polyclonal anti-Fab antibody specific for several of the primary antibodies on the beads*. The detaching beads are added to the bead-bound cells. The polyclonal antibody on the detaching beads competes with antibody/antigen binding at the cell surface and releases the antibody and beads from the cells. The target cells are left viable, unstimulated, and without antibody on their surface.

Purified cells obtained by these methods will be viable and can be used for functional studies as well as for cell culture.

For negative selection, the cell that needs to be depleted from a population is labeled and left in the column, while the eluate or the nonmagnetic fraction will contain all other cell types except the one that

was selected for adsorption. Magnetic cell enrichment and staining of disseminated tumor cells with antibodies directed against various epithelial antigens, such as EMA, pan-Cytokeratin, and CK-20, are used for molecular analysis. For enrichment of nonepithelial tumors, magnetic bead capture using anti-CD45 (the leukocyte common antigen marker) will remove much of the nontumor cell population. Sometimes density gradient separation followed by magnetic bead capture will enrich a very small population of cells.

It is important to realize that cell separation in a tube, not a column, gives a higher yield of viable cells and allows efficient separation of cells even in viscous samples. Thus, magnetic beads can be used to isolate cells from a wide range of starting materials.

It is not necessary to remove the magnetic beads from cells prior to isolation of nucleic acids. Follow procedures for isolating DNA or RNA from cells found in Sections 7.3.2 and 7.3.3.

7.1.4 Laser Capture Microdissection (LCM)

The LCM technique enables scientists to target and extract specific cells from a tissue sample. The basic principles of the method are relatively simple. The tissue sample is usually mounted on a glass slide, covered with a transparent film, and studied under a light microscope. Once researchers have identified cells of interest, they activate a focused infrared laser beam. The heat of the beam melts the plastic film, causing it to adhere to the targeted cells, which then can be lifted away, leaving the rest of the tissue section behind.

Commercially available systems today provide effective tools to capture cells and recover biomolecules. In brief, after locating cells of interest, an LCM cap is placed over the target area. Pulsing of the laser through the cap causes the thermoplastic film to form a thin protrusion that bridges the gap between the cap and tissue and adheres to the target cell. Introduction of the solid-state UV-light laser with the beam size of less than 1 μm is the latest advance in the LCM technique. Combined with other innovations, such as mounting of the tissue sample on a metal frame with a 0.9-μm PET-membrane instead of a glass slide, this system greatly improves the cutting performance. However, handling of the LCM cap from this slide is the same as from a regular glass slide. Thus, most of the LCM systems applied today remove the target cells by lifting the cap, with the exception of some newer systems that directly catapult the captured tissue sample into a collection device. Biomolecules are then extracted from the cells using standard nucleic acid extraction procedures (see below).

DNA and RNA can be extracted from microdissected cells using standard nucleic acid extraction procedures (see below). Genomic DNA can be successfully extracted from routinely processed, stained, and microdissected frozen, as well as formalin-fixed paraffin embedded tissue sections. Total and mRNA extraction from microdissected stained or unstained frozen sections has been demonstrated, using a variety of RNA isolation protocols and commercially available kits.

In general, long periods of tissue immersion in aqueous media for staining are deleterious for RNA, mainly due to activation of endogenous RNases. Good quality RNA can be obtained with shorter staining times for both routine histological stains and immunostaining, in conjunction with conventional protection against RNases, such as snap-freezing. In addition, formalin fixation performed by perfusion or by immersion of the tissue usually results in lower RNA recovery, when compared with methanol, acetone, and ethanol/acetone fixation.

The applications of LCM in medical research are rapidly widening; currently, they include:

- research of the relationship between genes and disease;
- cancer research via morphology and gene analysis;
- specific gene expression during their nascent period; and

- proteomics studies.

Consequently, molecular techniques that have been applied to cells captured by LCM include RT-PCR amplification, loss of heterozygosity (LOH), microsatellite instability analysis, differential gene profiling, and expression profiling using arrays for genomics, as well as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of protein products, Western blotting, immunoquantification, and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry for proteomics. LCM allows direct comparisons of gene amplification and expression with protein appearance in normal and diseased cells of the same tissue specimen. The microdissected cDNA libraries have been designed to approximate the true pattern of gene expression of pure cell populations of target cells in their actual tissue context. A combination of all mentioned analytic procedures with LCM has a potential to advance molecular diagnosis, assessment of prognosis, therapy selection, and monitoring of the therapy response in various clinical settings.

7.2 Pathogen Enrichment and Concentration Methods

The sensitivity that is generally achieved by amplification procedures in molecular assays does not generally warrant the need for enrichment or concentration of specimen. Extreme sensitivity in molecular assays can be attained by using "nested" amplification, whereby the nucleic acid is enriched in the first round of amplification and subsequently amplified by a second round of PCR. Sometimes enrichment in PCR refers to a process in which inhibitory substances that could possibly interfere with amplification are removed by specialized specimen processing protocols. (The role of inhibitory substances in PCR has been addressed in CLSI/NCCLS document MM3—*Molecular Diagnostic Methods for Infectious Diseases*.) In the laboratory, samples can be preprocessed for pathogen enrichment using a combination of gravity dispersion and centrifugation to separate obscuring blood, mucus, inflammation, and other debris that may interfere with molecular analysis. Depending on the application, one or more of these approaches can be used.

In some cases, however, one may need to concentrate the specimen in order to enrich for the pathogen or free circulating nucleic acid to be amplified. Enrichment of nucleic acids is also indirectly achieved when the DNA or RNA is extracted from target cells that have been selected or isolated by various methodologies. The nucleic acids or pathogens to be enriched could originate from blood, tissues, and cell-free systems such as body fluids, or from cytology specimens including fine needle aspirations. The requirement for concentration or enrichment depends on the diagnostic use, specimen type, pathogen factors, and often the specific disease syndrome.

7.2.1 Concentration of Nucleic Acids (DNA/RNA) in Cell-Free Biological Fluids

Increased amounts of circulating host DNA have been found in a variety of disorders including cancer, autoimmune disease, and infection.[47,48] Moreover, scant amounts of fetal DNA have been detected in the plasma/serum of pregnant women.[49] An evolution in molecular techniques has recently allowed much better characterization of circulating DNA in these conditions. Recent discoveries have brought a new understanding of circulating DNA and shown promise for the detection and follow-up of various disorders.

Concentration of nucleic acids can be achieved by centrifugation of plasma or cell-free fluids at high speeds or by ultrafiltration. Further concentration is possible by reducing the volume of the final diluent in which the nucleic acid is suspended after extraction (see Section 7.3).

7.2.2 Concentration of Pathogen in Serum and Plasma by High-Speed Centrifugation

When the numbers of organisms present in a specimen are low, it may be advantageous to enrich the test sample for the organism being quantitated or detected. This principle has been applied to some

quantitative assays that are used for the detection of human immunodeficiency virus and hepatitis B virus. An aliquot of serum or plasma (about 0.5 to 1.0 mL) is concentrated by high-speed centrifugation (approximately 24 000 x g for 60 minutes). High-speed centrifugation is used to pellet the virus, and the pellet is then resuspended in a small volume prior to testing. It should be noted that sample enrichment procedures may also concentrate proteins and other matrix material, which could serve to interfere with molecular test methods.

7.2.3 Concentration of Pathogen by Filtration

Concentration of pathogens using ultrafiltration devices to concentrate the solution is useful for detection of low numbers of microorganisms. Concentration is done either by vacuum elution of fluid or by centrifugal ultrafiltration. The devices use ultrafiltration and microfiltration membranes of varying porosity.

7.2.4 Concentration of Pathogens in CSF and Other Body Fluids Containing Cells

CSF, urine, bronchoalveolar lavage, vitreous fluid, culture fluids, and other body fluids may need to be treated differently, as they may possess inhibitory substances that could interfere with molecular assays. After these specimens are concentrated or enriched by centrifugation or filtration, the nucleic acids have to be extracted as described in Section 7.3.

7.2.5 Concentration of Pathogen in Fecal Specimens

Stool specimens first need to be diluted with buffered solution (pH 7.4) and centrifuged to get rid of debris. The specimen then undergoes filtration for removal of any cellular debris. The resulting filtrate will have mostly microorganisms. The filtrate can be concentrated by high-speed centrifugation as described above in Section 7.2.2. The nucleic acids have to be extracted using standard protocols as described in Section 7.3.

7.2.6 Fixation of Tissue and Biopsy Specimens

7.2.6.1 Formaldehyde (Formalin)

Formaldehyde as a 10% neutral buffered formalin (NBF) is the most widely used fixative. Even short-term treatment of tissue sections with formalin has been shown to decrease the DNA solubility.[50] Although some studies have shown yields of high molecular weight DNA from formalin-fixed tissue,[51] treatment of tissue with formalin generally induces considerable DNA degradation and is therefore not recommended for tissue slated for molecular analysis.

7.2.6.2 Other Tissue Fixatives

Glutaraldehyde:

Although widely used as a standard fixative for electron microscopy, the slow penetration and the need for periodic purification to maintain the functional aldehyde levels greatly limit the use of glutaraldehyde as a biological fixative.[52] However, it has been shown that 1% glutaraldehyde at pH 7.0 better preserves high molecular weight DNA, as compared to 10% formalin.[50]

Ethanol and Methanol:

One hundred percent ethanol and methanol are excellent fixatives for preserving both high molecular weight DNA and RNA, because they cause little chemical change of nucleic acids. Physical chemical

measurements have shown that DNA is largely collapsed in ethanol (65% v/v) and methanol, and there is substantial reversion to the original form when the denatured DNA is rehydrated.[53]

Carnoy's Fixative:

Carnoy's fixative is a mixture of ethanol (60%), chloroform (30%), and glacial acetic acid (10%). Well-preserved RNA was found to be more easily extractable from Carnoy's fixed tissue when compared to formalin-fixed tissue.[54]

Methacarn Fixative:

Methacarn fixative is a mixture of methanol (60%), chloroform (30%), and glacial acetic acid (10%). It has been shown that the extraction efficiency and integrity of total RNA from methacarn-fixed tissue are comparable to that from unfixed frozen cells.[55]

Acetone:

Acetone has been used as a fixative in the acetone-methylbenzoate-xylene (AMeX) technique,[56] which involves overnight fixation of tissue in acetone at -20 °C, then clearing in methylbenzoate and xylene before paraffin embedding. The AMeX method yields good quality high-molecular weight DNA, and mRNA can also be detected by dot-blot hybridization using RNA isolated from AMeX-fixed tissue.[57]

HOPE Fixation:

The Hepes-glutamic acid buffer-mediated organic solvent protection effect (HOPE) technique consists of incubation of fresh tissue in a protecting solution, comprising a mixture of amino acids at pH 5.8 to 6.4.[58] **NOTE:** The critical step is the overnight incubation with HOPE reagent at 2 to 8 °C. If RNA is the target molecule, one should be aware that cells are still alive during the initial HOPE incubation. This influences the RNA expression profile, making the method somewhat doubtful for RNA analysis. This is followed by incubation in acetone at 0 to 4 °C for dehydration, and subsequently tissue is embedded in paraffin. DNA and RNA derived from HOPE-fixed tissue are suitable for further molecular analysis by PCR, RT-PCR, and *in situ* hybridization.

Microwave (MW) Irradiation:

Electromagnetic waves with a frequency between 300 MHz and 300 GHz are classified as microwaves.[59,60] MW fixation depends on the chemical environment around the specimen during irradiation, duration of microwave exposure, and sequence of microwave irradiation and other chemical or physical fixation methods.[52] Thus, five MW fixation methods have been defined.[52]

- *Stabilization* wherein specimens are subjected to MW irradiation *in situ* or when immersed in a physiological salt solution in an attempt to preserve structures without the superimposed effects of a chemical fixative;

- *Fast or ultrafast primary MW chemical fixation* in which specimens are irradiated by MW energy in a chemical environment for a short period of time ranging from milliseconds to seconds;

- *MW irradiation followed by chemical fixation* involves continued immersion of specimens in a chemical fixative (such as formaldehyde) for minutes to hours after MW irradiation to improve uniformity of fixation;

- *Primary chemical fixation followed by MW irradiation* to facilitate cross-linking of fixatives within the specimen; and

- *MW irradiations in combination with freeze fixation* limit freezing artifacts.

In general, irradiation times less than 60 seconds, final irradiation temperature between 50 to 55 °C, and solution volume less than 50 mL in containers with at least one dimension that is ~1 cm are recommended.[61]

MW fixation in phosphate-buffered saline has been shown to be better than formalin fixation for the preservation of excellent quality genomic and viral DNAs.[62]

Household MW ovens have severe limitations in terms of safety and reproducibility. To overcome these limitations, experimental tools have been developed to standardize and calibrate MW ovens for tissue fixation. The neon bulb calibration tool maps regions of high and low power in an MW oven. Agar-saline-Giemsa tissue phantoms help determine the MW irradiation conditions that will uniformly heat a tissue.[61]

Formalin-Fixed Paraffin-Embedded Tissue (FFPET)

Formalin-fixed paraffin-embedded tissue (FFPET) is mainly used for determination of genotype in archived cancerous tissue. Archived FFPET is an enormous resource of DNA for cancer research. It is also used for determination of a genotype of a deceased individual with only an archived FFPET sample available. An FFPET sample is typically collected for retrospective analysis, or in a situation in which fresh or frozen tissue is not available. Typically, the tissue sample had not initially been intended for molecular analysis. An FFPET sample is also processed for nucleic acid analysis if histopathological analysis reveals unexpected findings, and makes subsequent nucleic acid analysis necessary for a more definitive diagnosis.

An FFPET sample can be easily handled and shipped at room temperature. For optimal performance of nucleic acid analysis, fresh tissue should be thinly sectioned (ideally, up to 0.2 cm in thickness, and not more than 0.3 cm in thickness, so that formalin can penetrate and fix tissue in a timely fashion) and fixed in neutral buffered formalin for not more than 12 hours. If thick tissue or a large specimen is to be fixed in formalin before thorough gross examination and tissue sampling for paraffin embedding, tissue can be incised so that formalin can enter the incision clefts and optimally fix tissue in a timely fashion. B-5 or other mercury-based fixative and decalcifying fixative significantly decrease quality and quantity of extracted DNA.

In general, an FFPET sample is not a suitable source for RNA or high quality genomic DNA (e.g., for Southern blotting). DNA in FFPET is partially degraded and fragmented due to formalin fixation. DNA segments of less than 200 bp can be amplified efficiently from FFPET samples. It is desirable to design primers, so an amplified segment is less than 200 bp or 100 bp, especially when the amount of DNA template is small. If no native material is available, FFPET may be used to extract RNA for subsequent RT-PCR; however, the size of the amplicon is recommended to be no more than 130 bp.[63]

The optimal thickness of FFPET sections for nucleic acid analysis depends on the size and cellularity of tissue. In general, 20 µm of large tissue (cross-section greater than 1 cm^2) or 40 to 80 µm of smaller tissue may be sufficient for PCR analyses. For DNA extraction, an FFPET block can be sectioned into thick sections (each 20 µm or more) and placed in a plastic tube, preferably in a screw-cap tube. A microtome blade should be cleaned by 100% ethanol between samples. Immediately prior to sectioning an FFPET block of interest, a blank paraffin block without any tissue should be sectioned at least twice and placed into two tubes, in order to monitor cleaning of the blade and avoid carry-over contamination from previous blocks. At least one of these tubes containing blank tissue sections should be processed to DNA extraction and tested in parallel with the sample of interest to ensure no carry-over contamination.

The tissue sections can then be deparaffinized by xylene, then incubated in ethanol, air-dried, and digested by proteinase K for subsequent DNA extraction. For manual dissection and DNA extraction, one tissue section can be stained with hematoxylin and eosin and coverslipped for marking areas of interest under a light microscope, and another hematoxylin and eosin-stained tissue section can be made for dissection of tissue. The blade or needle should be changed between samples to avoid contamination. For laser capture microdissection (LCM; see Section 7.1.4), a PET block should be sectioned (approximately 5 to 6 μm in thickness), deparaffinized, and stained with hematoxylin and eosin.

7.3 Nucleic Acid Preparation

7.3.1 Isolation of Viral Nucleic Acids

Viral genomes are highly variable in size and composition. Viral genomes can consist of either double- or single-stranded DNA or double- or single-stranded RNA. The genomic nucleic acids can be either linear or circular, and the virions themselves can vary greatly in size. The viral replication cycle of each virus varies; some viruses integrate into host genomes after infecting the cell, while other viruses replicate by insertion of the viral genome into different steps in the host cell cycle. Viruses can be isolated from either cellular or extracellular compartments of biological samples.

Integrated viral DNA is isolated using the same methods used for purification of genomic DNA, whereas intracellular viral RNA is isolated using the same methods used for RNA isolation. Similar techniques are used to isolate viral nucleic acid from viruses that have not integrated, but there are a number of additional considerations. These include the choice of starting material, the possible need to concentrate viral particles before isolation, and the methods used to lyse virus particles for release of their nucleic acid. In addition, some of the properties of viral nucleic acids lead to difficulties in isolation, such as their small size, and the presence in some viruses of tightly bound proteins.

Purification of viral nucleic acids can be challenging, because biological samples usually contain only low viral titers, but high levels of proteins and other contaminants. Purification methods should provide quantitative recovery of nucleic acids and complete removal of contaminants. In particular, many biological starting materials are rich in RNases; purification methods, therefore, require the use of efficient RNase inhibitors, such as chaotropic salts or other denaturing chemicals.

7.3.1.1 Methods

7.3.1.1.1 Organic Extraction Methods

Organic extraction can be used to purify chromosomal and viral nucleic acids from cell lysates, or pure viral nucleic acids from cell-free starting materials. Either phenol or a mixture of phenol-chloroform-isoamyl alcohol is used to separate nucleic acid-containing solutions into a nucleic acid-containing aqueous phase and a protein-containing organic phase. Nucleic acids are concentrated from the aqueous phase by alcohol precipitation.

A modified organic extraction method suitable for extraction of RNA was first described by Chomczynski and Sacchi.[64] This method, and modifications of it, have been widely used for purification of viral RNA. The method uses a mixture of mercaptoethanol and sarkosyl for cell lysis at pH 7.0, with guanidine thiocyanate for inactivation of contaminating RNases. Sodium acetate at pH 4.0 and water-saturated (i.e., acidic) phenol are then added, and RNA is precipitated with alcohol.

Some variations of the original organic extraction method for RNA purification have been developed. For example, a number of commercially available reagents use a monophasic solution of phenol and guanidine thiocyanate. Samples are homogenized and mixed with chloroform. The solution is separated

into organic and aqueous phases by centrifugation. RNA is precipitated from the aqueous layer by isopropyl alcohol, washed, and resuspended in RNase-free water.

7.3.1.1.2 Target Capture Method

In this method, target viral nucleic acids are hybridized to homologous capture probes that are linked to solid surfaces. Since specific capture probes must be developed for each virus that is to be tested, the method has a restricted number of applications. However, it is highly sensitive and specific, and is therefore well-suited for efficient and reliable high-throughput detection of frequently analyzed viruses.

A typical hybrid capture method for purification of viral DNA in serum samples employs biotinylated oligonucleotides that bind to streptavidin-coated magnetic beads. Serum samples are treated with guanidinium thiocyanate to inactivate DNases and RNases. Nucleic acids are mixed with biotinylated oligonucleotides specific to the target virus, heated to 96 ºC, chilled on ice, and then bound to streptavidin-coated magnetic beads. Contaminants are removed in multiple washing steps, which are performed using a magnetic tray. Protocols such as these are highly efficient for detection of specific viruses, and can also easily be automated for high-throughput needs.

7.3.1.1.3 Silica Technology

Silica particles were first used for isolation of nucleic acid from clinical samples by Boom et al.[65] Silica-based methods are simple, rapid, and reliable, and yield highly pure nucleic acids that can be used in sensitive downstream applications. Purification is based on adsorption of nucleic acid in cell lysates or cell-free samples to silica particles in the presence of high concentrations of chaotropic salts, such as sodium iodide, perchlorate, or guanidinium salts. The chaotropic salts in the lysis solution ensure denaturation and inactivation of RNases. The ionic strength and pH of the lysis and binding solution can be adjusted, so DNA or RNA is selectively bound, while cellular proteins and metabolites remain in solution. Finally, nucleic acid is eluted under low-salt conditions.

A number of commercial kits are available for isolation of viral nucleic acids using silica technology. Samples are first lysed under highly denaturing conditions, buffering conditions are adjusted, and samples are applied to the purification column containing the silica membrane. Nucleic acids bind to the membrane, and contaminants are removed in washing steps. Viral nucleic acids are eluted in suitable buffer, ready for direct use or safe storage.

7.3.1.2 Concentration of Starting Material

Many clinical starting materials, including stool, plasma, urine, cerebrospinal fluid, and other body fluids, often contain very low numbers of cells or viruses. In these cases, concentrating samples before beginning purification (usually by centrifugation or filtration) is important to improve yields.

7.3.1.2.1 Centrifugation

Centrifugation is generally used to concentrate viral nucleic acids in body fluids, and microconcentrators are commercially available for this purpose. After concentration, standard isolation procedures can be followed. When isolating viral nucleic acids from urine, specialized buffers containing chaotropic salts must be used at the beginning of the isolation procedure after concentration, as urine contains numerous unidentified PCR inhibitors.

Ultracentrifugation can be used to improve detection sensitivity of viral genomes in plasma. The addition of a centrifugation step prior to RNA extraction generates a pellet of viral particles. The pellet is redissolved either in PBS, or a suitable lysis buffer and viral nucleic acids are purified using one of the purification methods.

7.3.1.2.2 Filtration

Filtration is used to concentrate viral nucleic acids in stool specimens. Stool specimens are resuspended in sodium chloride solution, and the solution is clarified by centrifugation. The suspension is filtered through a 0.22-µm filter to remove cells from the specimen, eliminating cellular DNA from the preparation. Concentrated viral nucleic acids can then be isolated from the filtrate.

Filtration and centrifugation through a microconcentrator have been used to screen water samples containing very low viral titers.[66] Although water is monitored routinely for bacterial contamination, it is not usually tested for viral contamination, largely because the methods usually applied for such testing are not sufficiently sensitive, and are complex and expensive.

7.3.1.2.3 Cationic Detergents

Starting materials can be mixed with cationic detergents at the beginning of nucleic acid isolation procedures, providing both concentration and stabilization of nucleic acids. These effects result from the formation of complexes between the cationic heads of detergent molecules and negatively charged nucleic acid backbones. The hydrophobic detergent tails remain on the outside of the complexes, leading to precipitation and concentration of nucleic-acid-detergent complexes. Proteins do not bind to the detergents and remain in solution, so the use of detergents leads to removal of some contaminants and provides some nucleic acid stabilization. However, starting materials that contain high levels of protein, such as blood, may require additional methods for full stabilization.

After concentration and stabilization with cationic detergents, nucleic acids must be released from the charged complexes. When isolating viral nucleic acids, this is usually achieved either by organic extraction or by silica purification methods.

7.3.2 Isolation of Genomic DNA (Mammalian and Eukaryotic)

7.3.2.1 General Considerations for Isolation of Genomic DNA

Methods for isolating DNA from animal, human, yeast, and bacterial cell lysates are essentially the same as those for DNA isolation from tissue samples, described above. Different starting materials, however, have different characteristics that affect DNA isolation, particularly sample disruption and lysis, and removal of specific cell constituents. In addition, the quality of the sample and the way it was stored affect the quality and yield of the isolated DNA. The following are general considerations for isolation of genomic DNA; specific considerations for different sample types are discussed later in this section.

7.3.2.2 Starting Material

The experimental design often dictates what kind of starting material is used; that is, whether fresh, stored (frozen or preserved in some kind of fixative), or forensic samples are used, as well as the tissue type and age. The quality of the starting material affects the quality and yield of the isolated DNA. DNA yields will generally decrease if samples are stored under improper conditions, and the isolated DNA may be degraded. In addition, repeated freezing and thawing of samples should be avoided, as this will lead to genomic DNA of reduced size, and in clinical samples, to reduced yields of pathogen DNA (e.g., viral DNA).

DNA yields also depend on the size, type, and age of the starting material. The nucleic acid content of the cell type used also affects yields: a tissue comprised of small cells will have a higher cell density, and therefore is likely to contain more DNA than a sample of the same size comprised of larger cells. DNA content also depends on the haploid genome size and the ploidy of the cells.

7.3.2.3 Sample Disruption and Lysis

Complete sample disruption and cell lysis is essential for release of DNA from tissue samples. Insufficient disruption and lysis will lead to low DNA yields. Disruption generally involves use of a lysis buffer that contains a detergent (for breaking down cellular membranes) and a protease (for digestion of protein cellular or pathogen components).

Some sample types require additional treatment for efficient lysis. A guide to mechanical disruption methods is given in this section; specific recommendations for different sample types are discussed later in this section.

7.3.2.3.1 Homogenization Using a Syringe and Needle

Cell and tissue lysates can be homogenized using a syringe and needle. High-molecular-weight DNA can be sheared by passing the lysate through a 20-gauge (0.9 mm) needle, attached to a sterile plastic syringe, at least five to ten times or until a homogeneous lysate is achieved. Increasing the volume of lysis buffer may be required to facilitate handling and minimize sample loss.

7.3.2.4 Methods

The analysis of complex organisms through the application of molecular biology techniques requires the purification of pure, high-molecular-weight genomic DNA. Different technologies and methods are available for isolation of genomic DNA. In general, all methods involve disruption and lysis of the starting material, followed by removal of proteins and other contaminants, and subsequent recovery of the DNA.

7.3.2.4.1 Organic Extraction Methods

Organic extraction is a classical technique that uses organic solvents to extract contaminants from cell lysates.[67,68] The cells are lysed using a detergent, and then mixed with phenol, chloroform, and isoamyl alcohol. The correct salt concentration and pH must be used during extraction to ensure that contaminants are separated into the organic phase and that DNA remains in the aqueous phase. DNA is usually recovered from the aqueous phase by alcohol precipitation. DNA isolated using this method may contain residual phenol and/or chloroform, which can inhibit enzyme reactions in downstream applications, and therefore may not be sufficiently pure for sensitive downstream applications such as PCR.[69] Furthermore, this is a fairly time-consuming and technique-sensitive method that uses toxic compounds, and may not give reproducible yields.[70]

7.3.2.4.2 Salting-Out Methods

“Salting-out” is another technique in which proteins and other contaminants are precipitated from the cell lysate using high salt concentrations.[71] The precipitates are removed by centrifugation, and the DNA is recovered by alcohol precipitation. Removal of proteins and other contaminants using this method may be inefficient; and RNase treatment, dialysis, and/or repeated alcohol precipitation are often necessary before the DNA can be used in downstream applications. DNA yield and purity are often variable using this method.

7.3.2.4.3 Cesium Chloride Density Gradients

Genomic DNA can be purified by centrifugation through a cesium chloride density gradient. Cells are lysed using a detergent, and the lysate is alcohol precipitated. Resuspended DNA is mixed with CsCl and ethidium bromide and centrifuged for several hours. The DNA band is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover

the DNA. This method allows the isolation of high-quality DNA, but it is time consuming, labor intensive, and expensive, making it inappropriate for routine use.

7.3.2.4.4 Silica-Based Methods

Silica-based technology provides a simple method for isolation of high-quality DNA. This method is based on the selective absorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts, such as guanidine hydrochloride, guanidine isothiocyanate, sodium iodide, and sodium perchlorate.[65] Use of optimized buffers in the lysis procedure ensures that only DNA is adsorbed while RNA, cellular proteins, and metabolites stay in solution. These contaminants are washed away, and then high-quality DNA is eluted from the silica particles or membranes using a low-salt buffer. The eluted DNA is ready for use in most downstream applications.

Several companies offer kits for isolation of DNA based on silica technology. Specific procedures for DNA purification (as well as the resulting quality of purified DNA) differ depending on which type of kit is used. For example, silica-gel particles can be used either in suspension or as a membrane in spin columns or multiwell units designed for high-throughput procedures, including automated procedures. Use of spin columns is generally more convenient and avoids silica particle carry-over in the eluted DNA. Alternatively, use of magnetic beads coated with silica particles allows isolation of genomic DNA without centrifugation or vacuum processing.

The average size of genomic DNA isolated using silica-gel-membrane technology is 20 to 50 kb. DNA of this length is particularly suitable for PCR analysis as well as Southern blot analysis. However, this technique is not suitable if high-molecular-weight genomic DNA (DNA fragment size >100 kb) is required (see Section 7.3.2.4.5).

7.3.2.4.5 Anion-Exchange Methods

Solid-phase anion-exchange chromatography is based on the interaction between the negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. DNA binds to the substrate under low-salt conditions; impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers; and then pure DNA is eluted using a high-salt buffer. The eluted DNA is recovered by alcohol precipitation and is suitable for all downstream applications, including sensitive applications, such as transfection, microinjection, sequencing, and gene therapy.

Anion-exchange technology yields DNA of a purity and biological activity equivalent to at least two rounds of purification in CsCl gradients, but in much less time. This technology also completely avoids the use of toxic substances, and can be applied for different throughput requirements, as well as for different scales of purification. The isolated DNA is sized up to 150 kb. Several companies offer kits for isolation of DNA based on anion-exchange technology. Kit procedures from different companies vary in processing times and the quality and size of the isolated DNA.

7.3.2.4.6 Filter Paper-Based Methods

Use of filter paper blood collection devices provides a convenient way to store dried biological specimens and then isolate DNA from the samples for use in PCR. There is a large body of literature that discusses PCR methods using DBS on untreated filter paper for newborn screening. The specimen is simply spotted onto the paper and dried. The specimen can be washed and the DNA eluted; or the specimen can be fixed with methanol and the DNA eluted[72]; or there are commercially available kits to extract DNA from DBS on untreated filter paper.

Alternatively, treated filter paper impregnated with various compounds for DNA stabilization and isolation can also be used.[73] The filter paper contains compounds that lyse biological samples and bind

nucleic acids, as well as compounds that kill microorganisms and inhibitory nonmicrobial degradation of DNA (e.g., oxidation). Dried samples can also be stored at room temperature for many years without loss in genomic DNA integrity. In a study comparing four filter papers, both treated and untreated paper were found to be adequate for banking of DNA in DBS for at least 19 months at ambient temperature.[23]

7.3.2.5 Removal of RNA

Depending on the DNA isolation method used, RNA will be copurified with the DNA. RNA may inhibit some downstream applications, but it will not inhibit PCR. Treatment with RNase A will remove contaminating RNA; this can either be incorporated into the purification procedure or performed after the DNA has been purified. Prior to use, ensure that the RNase A solution has been heat-treated to destroy any contaminating DNase activity. Alternatively, use DNase-free RNase, which is commercially available.

7.3.2.6 Isolation of Genomic DNA From Animal and Human Tissue

7.3.2.6.1 Disruption of Animal and Human Tissue

Most animal and human tissues can be efficiently lysed using lysis buffer and protease or proteinase K. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Mechanical disruption using a homogenizer, a mixer mill with 3- to 7-mm stainless steel beads, or mortar and pestle prior to lysis can reduce lysis time. Skeletal muscle, heart, and skin tissue have an abundance of contractile proteins, connective tissue, and collagen, and care should be taken to ensure complete digestion with protease or proteinase K.

7.3.2.6.1.1 Disruption Using Rotor-Stator Homogenizers

Rotor-stator homogenizers thoroughly disrupt animal and plant tissues in 5 to 90 seconds, depending on the toughness of the sample. The rotor turns at very high speed, causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, by keeping the tip of the homogenizer submerged, and by holding the immersed tip to one side of the tube. Rotor-stator homogenizers are available in different sizes and operate with differently sized probes. Probes with diameters of 5 mm and 7 mm are suitable for volumes up to 300 µL and can be used for homogenization in microfuge tubes. Probes with a diameter of 10 mm or more require larger tubes.

7.3.2.6.1.2 Disruption Using Bead Mills

Cells and tissues can be disrupted using a mixer mill by rapid agitation in the presence of beads and lysis buffer. Disruption occurs by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the size and composition of beads, the speed and configuration of agitator, the ratio of buffer to beads, the disintegration time, and the amount of starting material. Disruption parameters must be determined empirically for each application.

7.3.2.6.1.3 Disruption of Tissue Samples Using a Mortar and Pestle

For disruption using a mortar and pestle, samples should be frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. For a typical protocol, see Appendix A.

7.3.2.7 Isolation of Genomic DNA From Blood

Blood samples are routinely collected for clinical analysis. Blood contains a number of enzyme inhibitors that can interfere with downstream DNA analysis. In addition, common anticoagulants, such as heparin

and EDTA, can interfere with downstream assays. DNA isolation from blood therefore requires a method to provide high-quality DNA without contaminants or enzyme inhibitors.

7.3.2.7.1 Removal of Erythrocytes From Mammalian Blood Samples

While erythrocytes (red blood cells) from birds, fish, and frogs contain nuclei and hence genomic DNA, mature erythrocytes from mammals do not. Since healthy mammalian blood contains approximately 1000 times more erythrocytes than nuclei-containing leukocytes (white blood cells comprising lymphocytes, monocytes, and granulocytes), removing the erythrocytes prior to DNA isolation can give higher DNA yields. This can be accomplished by several methods. One is selective lysis of erythrocytes, which are more susceptible than leukocytes to hypotonic shock and burst rapidly in the presence of a hypotonic buffer.[74] Alternatively, density-gradient centrifugation can be performed to recover mononuclear cells (lymphocytes and monocytes) and remove erythrocytes[75] (see Section 7.1.1). This technique also removes granulocytes. A third method is to prepare a leukocyte-enriched fraction of whole blood, called buffy coat, by centrifuging whole blood at 3300 x g for ten minutes at room temperature. After centrifugation, three different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat; and the bottom layer contains concentrated erythrocytes.[76]

7.3.2.7.2 Disruption of Blood Samples

Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using lysis buffer and protease or proteinase K.

7.3.2.7.3 Methods for Isolation of DNA From Blood Samples

As mentioned above, DNA isolation from blood requires a method to remove both intrinsic contaminants and those derived from anticoagulants, hence providing high-quality DNA. Most homemade methods for mammalian blood samples use a buffy coat preparation, from which DNA can be isolated in the same way as from animal and human tissues (see Section 7.3.2.3).

Silica membrane based kits for isolation of high-quality DNA from whole blood samples are available from commercial manufacturers. In these methods, separation of leukocytes is not necessary. Different kits are available for different scales and throughput requirements, as well as for automated procedures.

7.3.2.8 Isolation of Genomic DNA From Other Clinical Samples

In addition to blood, other biological fluids, swabs, and stool specimens are routinely collected for clinical analysis. Some of these, particularly stool, contain high levels of inhibitors and like blood DNA isolation methods, require methods that provide high-quality DNA without contaminants or enzyme inhibitors. Commercially available kits provide dedicated reagents that efficiently absorb inhibitory compounds in stool samples for efficient removal by centrifugation. Most other biological fluids can be treated in the same way as blood samples for isolation of DNA.

7.3.2.9 Isolation of DNA From Pathogens

Viral, fungal, parasite, and bacterial DNA can be isolated from clinical samples. Integrated viral (proviral) DNA is prepared using the same procedure as isolation of genomic DNA from the relevant sample. Viral DNA from virus particles is typically isolated from cell-free body fluids, such as plasma and CSF, in which viral titer can be very low. Virus particles may need to be concentrated before DNA isolation by ultracentrifugation, ultrafiltration, or precipitation. Addition of carrier DNA may also be necessary during DNA isolation when the expected yield of DNA is low. It should be noted that pathogen DNA or RNA will be mixed with host nucleic acids, unless specific isolation procedures are used.

To isolate bacterial DNA from biological fluids, the bacterial cells should be pelleted, and the DNA isolated as for bacterial cell cultures (see below).

7.3.2.10 Isolation of Genomic DNA From Animal, Human, Yeast, and Bacterial Cell Cultures

Animal and human cell cultures can be efficiently lysed using lysis buffer and protease or proteinase K. Yeast cell cultures must first be treated with lyticase or zymolase to digest the cell wall. The resulting spheroplasts are collected by centrifugation and then lysed using lysis buffer and proteinase K or protease. Yeast cells (and other unicellular animals) can alternatively be disrupted using a mixer with 0.5-mm glass beads prior to lysis. Many bacterial cell cultures can also be efficiently lysed using lysis buffer and protease or proteinase K. Some bacteria, particularly gram-positive bacteria, require preincubation with specific enzymes (e.g., lysozyme or lysostaphin) to lyse the rigid, multilayered cell wall. Bacterial cells can also be disrupted using a mixer mill with 0.5-mm glass beads prior to lysis. It is essential that glass beads are pretreated by washing in concentrated nitric acid.

7.3.3 Isolation of RNA

7.3.3.1 Disruption and Homogenization of Starting Materials for Isolation of RNA

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA isolation procedures. Following disruption and homogenization, RNA purification is carried out using one of the methods described below.

Disruption and homogenization are generally carried out in the presence of an organic solvent or a strong chaotropic agent in order to inhibit endogenous RNases that are released during the process. Nonetheless, changes in the RNA expression pattern can occur prior to or during disruption and homogenization. For accurate gene-expression analysis, the sample should be stabilized first (see Section 6.4.9.2). In the case of small samples, the addition of RNase inhibitors may be necessary.

Some disruption methods simultaneously homogenize the sample while others require an additional homogenization step. The different disruption and homogenization methods are described in more detail below.

7.3.3.1.1 Disruption and Homogenization Using Rotor-Stator Homogenizers

Rotor-stator homogenizers thoroughly disrupt and simultaneously homogenize, in the presence of lysis buffer, animal tissues in 5 to 90 seconds, depending on the toughness of the sample. Rotor-stator homogenizers can also be used to homogenize cell lysates (see Section 7.3.2.6.1.1). The rotor turns at a very high speed, causing the sample to be disrupted and homogenized by a combination of turbulence and mechanical shearing. Foaming of the sample should be kept to a minimum by using properly sized vessels, by keeping the tip of the homogenizer submerged, and by holding the immersed tip to one side of the tube. **NOTE:** By using disposable tips and rigorous cleaning procedures of the homogenizer, the risk of sample-to-sample carry-over can be reduced.

7.3.3.1.2 Disruption and Homogenization Using Bead Mills

In bead milling, cells and tissue can be disrupted by rapid agitation in the presence of beads and lysis buffer. Disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by size and composition of beads, speed and configuration of agitator, ratio of buffer to beads, disintegration time, and amount of starting material.

The optimal beads to use are 0.1-mm (mean diameter) glass beads for bacteria, 0.5-mm glass beads for yeast and unicellular animal cells, and 3- to 7-mm stainless steel beads for animal and plant tissues. Glass beads must be pretreated by washing in concentrated nitric acid. Alternatively, acid-washed glass beads can be purchased from a number of suppliers. Plant material (as well as the beads and disruption vessels) must be precooled in liquid nitrogen, and disruption should be performed without lysis buffer. All other disruption parameters must be determined empirically for each application.

7.3.3.1.3 Disruption Using a Mortar and Pestle

For disruption using a mortar and pestle, freeze the sample immediately in liquid nitrogen and grind to a fine powder under liquid nitrogen. Transfer the suspension (tissue powder and liquid nitrogen) into a liquid-nitrogen-cooled, appropriately sized tube and allow the liquid nitrogen to evaporate without allowing the sample to thaw. Add lysis buffer and continue as quickly as possible with the homogenization according to one of the methods below. Note that grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization must be performed separately before proceeding.

7.3.3.1.4 Homogenization Using Spin-Column Homogenizers

Some companies offer spin-column homogenizers in combination with silica-membrane technology. This is a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of the samples. The lysate is loaded onto the spin column sitting in the collection tube, centrifuged, and the homogenized lysate is collected.

7.3.3.2 Methods of Isolation

Different technologies and methods are available for isolation of RNA and/or cleanup of RNA from enzymatic reactions. In general, the methods involve disruption and lysis of the starting material, followed by removal of proteins, DNA, and other contaminants. In this section, different individual techniques are described. Typical protocols use a combination of several of these techniques. The choice of a particular protocol depends on the type of RNA (total RNA, cytoplasmic RNA, mRNA, low molecular weight RNAs), the purity needed for downstream applications, the desired time and expense per sample, and whether intact RNA is essential or not.

7.3.3.2.1 Enzymatic Digestion

A simplified method for isolation of RNA from cells involves cell lysis and digestion of proteins with proteinase K in the presence of sodium dodecyl sulfate (SDS) and an RNase inhibitor. After inactivation of the proteinase K, by heating or organic extraction, the contaminating genomic DNA is then digested with DNase I for 30 minutes to one hour. Contaminants in the lysate may inhibit downstream applications or lead to RNA degradation. This method cannot be used with tissues, since lysis with proteinase K is relatively slow and inefficient, and it is difficult to prevent endogenous RNases from degrading the RNA. Alternatively, a disruption under chaotropic conditions can be performed; subsequent dilution of the lysate to a salt concentration tolerated by enzymes like proteinase K can increase the yield of RNA without degradation during the enzymatic treatment.

Digestion with DNase I is often used at the end of a protocol to remove any DNA. Following DNase treatment, the DNase can be removed by any of the techniques that separate proteins from nucleic acids, including organic extraction followed by alcohol precipitation or cleanup using silica-based or anion-exchange methods. Complete removal of DNase I is absolutely essential if RNA shall be used for RT-PCR analyses. Any residual DNase I can degrade cDNA during cDNA synthesis and can severely impair cDNA generation and quantity.

7.3.3.2.2 Organic Extraction

Organic extraction is a traditional technique that is often combined with proteinase K digestion, extraction with strong denaturants, alcohol or LiCl precipitation, and/or CsCl density gradients. Typically, the sample is mixed with phenol at acid pH. The phenol lyses cells and denatures the proteins in the sample. At acid pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA remains charged and partitions into the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, and then with chloroform to extract the remaining phenol. RNA in the aqueous phase is then precipitated with ethanol or isopropanol and/or separated on a CsCl gradient.

RNA isolated using organic extraction may contain residual phenol and/or chloroform, which can inhibit downstream reactions, such as RT-PCR, and can affect absorbance readings.[77] The use of toxic and teratogenic reagents is a disadvantage. In addition, the method is time-consuming and requires manual skill to transfer the aqueous phases.

7.3.3.2.3 Extraction With Strong Denaturants

Chaotropic agents, such as guanidine isothiocyanate and guanidine hydrochloride, are strong denaturants that immediately inactivate RNases to ensure isolation of intact RNA. Guanidine salts are also sufficient to disrupt cells. Extraction with chaotropic reagents is typically combined with subsequent organic extraction, alcohol or LiCl precipitation, CsCl density gradients, silica-based methods, anion-exchange methods, and/or hybrid selection.

7.3.3.2.4 Alcohol and LiCl Precipitation

Both alcohol and LiCl precipitation rely on “salting out” nucleic acids. Precipitation with ethanol or isopropanol, in the presence of sodium or ammonium acetate, is included in many RNA isolation protocols. The RNA must be partially purified before precipitation, since proteins and DNA also precipitate. The precipitate is then washed with 70% alcohol to remove the remaining salts, dried, and redissolved. This technique allows concentration of the RNA and removal of salts, but it is time-consuming. In general, this method works best with larger amounts of RNA. The precipitated pellet can be lost when removing the alcohol or during the drying procedure. This can occur with low amounts of RNA, when the pellet is very small, or when residual organic solvents (such as chloroform) partition to the bottom of the centrifuge tube with the RNA pellet.

LiCl can be used to differentially precipitate RNA while DNA remains in solution. LiCl precipitation provides higher purity than typical organic extraction methods. However, it typically takes several hours, taking more time than alcohol precipitation. Furthermore, precipitation requires high molar concentrations of LiCl, which can interfere with downstream enzymatic reactions, such as RT-PCR. LiCl precipitation is usually followed by alcohol precipitation to remove LiCl from the sample.

7.3.3.2.5 CsCl and Sucrose Density Gradients

RNA can be purified by centrifugation through a CsCl density gradient. The partially purified RNA is mixed with CsCl and ethidium bromide and centrifuged at a high *g*-force (e.g., 36 000 to 40 000 x *g*) for several hours, usually overnight (12 to 24 hours). RNA pellets to the bottom of the centrifuge tube, while DNA and proteins remain in the supernatant. The RNA pellet is collected and reprecipitated by alcohol precipitation to remove the remaining CsCl. This method allows isolation of high-quality RNA, but it is very time-consuming, labor-intensive, and expensive, making it difficult to use for preparation of multiple samples. The use of toxic ethidium bromide and CsCl provide a further disadvantage.

Small RNA species, such as tRNAs and 5S rRNAs, do not pellet in CsCl gradients. These small RNA species can be isolated by fractionation through sucrose gradients or agarose gels containing methylmercuric hydroxide. However, methylmercuric hydroxide is volatile and extremely toxic, making it hazardous to use and difficult to dispose of the associated chemical waste.

7.3.3.2.6 Anion-Exchange Chromatography

Solid-phase anion-exchange chromatography is based on the interaction between the negatively charged phosphates of the nucleic acid and positively charged surface molecules on the substrate. RNA binds to the substrate under defined salt conditions, and other contaminants, such as DNA, cellular proteins, and metabolites are eluted with different salt concentrations. This allows parallel purification of RNA, DNA, and low-molecular-weight RNA species from the same sample. The eluted nucleic acids are recovered by alcohol precipitation and are suitable for all downstream applications.

Anion-exchange technology yields RNA of a purity and biological activity equivalent to at least two rounds of purification in CsCl gradients, but in much less time. The isolated RNA also includes small RNA species that are excluded when using CsCl gradients or silica-based methods, and these small RNA species can be selectively isolated. In addition, this technology completely avoids the use of toxic substances, and can be applied for different throughput requirements, as well as for different scales of purification. Some companies offer kits for isolation of RNA and DNA based on anion-exchange technology. Kit procedures from different companies differ in their processing times and the quality and size of the isolated RNA.

7.3.3.2.7 Silica-Based Methods

Silica-based technology provides a reliable and fast method for isolation of RNA. This method is based on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts, such as guanidine hydrochloride, guanidine isothiocyanate, sodium iodide, and sodium perchlorate. The use of specific buffers in the lysis procedure ensures that only RNA is adsorbed while DNA, cellular proteins, and metabolites remain in solution. These contaminants are washed away, and high-quality RNA is eluted from the silica using a low-salt buffer. With use of the appropriate materials or kit, the eluted RNA can then be ready for use in most downstream applications. **NOTE:** In samples with low RNA content, DNA may be adsorbed instead of RNA. Testing for contaminations of RNA with genomic DNA may be necessary in downstream experiments.

Several technologies based on silica are used in the field, which differ in procedure and quality of the isolated RNA. For example, silica-gel particles can be used either in suspension or as a membrane in spin columns or multiwell units designed for high-throughput procedures, including automated procedures. Use of spin columns is generally more convenient and avoids silica particle carryover in the eluted RNA.

7.3.3.2.8 Isolation of mRNA Using Oligo(dT) Affinity Chromatography

The discovery that eukaryotic mRNA contains covalently attached sequences of polyadenylic acid [poly(A)] at the 3′ end[78] led to the use of mRNA affinity purification procedures, which utilize the hybridization of poly(A) sequences to oligo(dT) sequences immobilized on a solid matrix. Early methods incorporated oligo(dT) sequences covalently attached to cellulose.[79] In addition to those containing oligo(dT) cellulose, commercially available kits also employ oligo(dT) attached to magnetic particles, polystyrene-latex beads, or polystyrene microtiter plate wells. Typically, total mRNA is isolated and purified from the clinical specimen and prepared for affinity purification by heating at 65 ^{o}C for five minutes followed by rapid cooling on ice to eliminate secondary structure. The prepared RNA is then applied to the immobilized oligo(dT) matrix, the matrix is washed several times to remove nonspecifically bound material, and the mRNA is eluted. The eluate can then be concentrated by

precipitation with ethanol or by evaporation in a centrifugal vacuum concentrator. Oligo(dT) affinity purification methods are not suitable for purifying nonpolyadenylated (e.g., bacterial) mRNA.

7.3.3.3 Special Considerations for Isolation of RNA From Different Sample Sources

Some sample sources have differences in their RNA or contain substances that can cause problems in RNA isolation and analysis. These sample sources require special considerations, which are generally not necessary when working with "standard" sample sources (e.g., cell cultures, liver, kidney). In this section, considerations for working with a number of different sources are discussed.

7.3.3.3.1 Heart, Muscle, and Skin Tissue

RNA isolation from skeletal muscle, heart, and skin tissue can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. In order to remove these proteins, which can interfere with RNA isolation, the sample needs to be treated with a protease. However, the protease digest needs to be carried out under conditions that do not allow RNA degradation (see Section 7.3.3.2.1).

7.3.3.3.2 Blood

Blood samples are routinely collected for clinical analysis. Blood contains a number of enzyme inhibitors that can interfere with downstream RNA analysis. In addition, common anticoagulants, such as heparin and EDTA, can interfere with downstream assays. RNA isolation from blood requires a method to provide high-quality RNA without contaminants or enzyme inhibitors.

Erythrocytes (mature red blood cells) may be less important for RNA isolation than reticulocytes (immature red blood cells), which contain mRNA (mostly globin mRNA). The target of RNA isolation from whole blood is primarily leukocytes (white blood cells). Leukocytes consist of three main cell types: lymphocytes, monocytes, and granulocytes.

Removing the erythrocytes may simplify RNA isolation. This can be accomplished by selective lysis of erythrocytes, which are more susceptible than leukocytes to hypotonic shock and burst rapidly in the presence of a hypotonic buffer. The intact leukocytes are then collected by centrifugation, and RNA isolation is carried out as with other eukaryotic cells. A common alternative to erythrocyte lysis is density-gradient centrifugation. In contrast to erythrocyte-lysis procedures, density-gradient centrifugation only recovers mononuclear cells (lymphocytes and monocytes) and removes granulocytes. Mononuclear cells isolated by density-gradient centrifugation can then be processed for RNA isolation using the same methods as with other cell types. It should be noted, however, that gene transcription profiles change markedly in stored whole blood specimens if steps are not taken to stabilize the specimen immediately after phlebotomy.[20] Stabilizing reagents specific for whole blood samples are commercially available and should be used if the stability of mRNA and rRNA are desired.

7.3.3.3.3 Bacteria

Bacterial mRNAs differ from eukaryotic mRNAs in a number of essential features. Prokaryotic mRNAs have no 5′ cap and only rarely have poly(A) tails. The absence of a poly(A) tail means that mRNA isolation by oligo(dT) hybrid selection is not possible. In addition, oligo(dT) primers cannot be used to prime first-strand cDNA synthesis, so random primers must be used.

In addition, bacterial mRNAs are highly unstable, with an average half-life of about three minutes for fast-growing bacteria. Sometimes the bacterial mRNA begins to degrade while it is still being translated. For this reason, gene-expression studies are even more difficult to conduct with prokaryotes than with eukaryotes. To accurately preserve gene-expression patterns and to maximize the amount of fully intact

mRNA isolated, samples must be stabilized immediately after sample harvesting and prior to sample processing (see Section 6.3).

7.3.3.3.4 Free Circulating RNA in Serum

RNA associated with proteolipids has been detected in the serum of some cancer patients. The concentration is approximately tenfold higher than free circulating DNA in human plasma, and the RNA is relatively stable, with a half-life of about two days in human whole blood. Nonetheless, the RNA can be degraded by repeated freeze-thaw cycles. As with viral RNA in cell-free body fluids, addition of carrier RNA may be necessary during RNA isolation of this RNA.

7.3.3.3.5 Fatty Tissues

RNA isolation from fatty tissues (e.g., adipose tissue, brain) can be difficult with aqueous lysis buffers (often used in commercial RNA extraction methods), due to the interference of the lipid content. Tissues with high lipid content are not completely lysed with aqueous lysis buffers, leading to low yields and in the case of high amounts of tissue, to membrane clogging. Therefore, organic extraction of fatty tissues is necessary, since phenol is able to lyse lipid-containing samples completely.

A combination of organic extraction and silica-based membrane technology avoids the main disadvantages of organic extraction described above, such as residual phenol and chloroform in the RNA, as well as the risk of losing RNA at the precipitation step. The tissue is homogenized in phenol or mixture of phenol and GTC, followed by phase separation mediated by chloroform. After adjusting binding conditions, the aqueous phase can directly be loaded onto a silica membrane, followed by the usual purification steps.

8 Sample Storage

Sample is a product derived from the specimen. A number of samples can be obtained from a tube of blood, including single- or double-spin plasma, PBMCs, cell pellet, whole blood pellet, buffy coat, etc. Nucleic acids are then extracted from these samples for use in various molecular diagnostic test methods. Cell isolation or enrichment methods chosen, along with activation state and cell differentiation stage, will affect the quantity and quality of nucleic acids extracted from blood samples. **NOTE:** Neutrophils express low levels of mRNA while having the majority of genomic DNA of a whole blood sample. PBMCs obtained by gradient centrifugation may have significantly decreased DNA yields, because neutrophils are essentially eliminated.

8.1 Storage Requirements—DNA

DNA is a relatively stable macromolecule, and its extraction from blood is successful even when blood is stored at room temperature for up to eight days. Once isolated, DNA is stable at 2 to 8 ^{o}C for at least one year. Generally, DNA is stored in solution. In fact, distilled water can be used if DNA is to be used for PCR and/or endonuclease digestion within a few days after its isolation. Tris/EDTA (pH of 7.2), however, is considered the preferred buffer for DNA storage, because buffering limits pH variations, which can occur in water and can subsequently lead to spontaneous degradation of DNA. Nucleic acids retain recognizable characteristics during long-term storage when they are frozen in solution. It is recommended that the DNA solution be stored as a primary stock solution frozen at -70 ^{o}C or lower. Alternatively, if the sample is required for multiple testing, the DNA sample can be frozen in multiple aliquots at -70 ^{o}C or lower for subsequent analysis in order to avoid repeated freeze-thaw cycles of the stock solution. Aliquoting the sample also minimizes the possibility of sample contamination, which will result in analytical inaccuracy.

8.2 Sample Storage Requirements—RNA

RNA is an extremely labile macromolecule. Purified RNA is best stored as a precipitate in ethanol at -70 ^{o}C or lower. To store purified RNA, use sterile, hydrophobic, plastic tubes that have not been handled with ungloved hands and that have been treated with diethylpyrocarbonate water to rid the tubes of RNases, which are very stable. Alkaline pH (7.1 to 7.5) is much more effective than acidic or neutral conditions for preservation of RNA samples. Purified RNA is stable for as long as three hours following the first freeze-thaw cycle. Repetitive freeze-thaw cycles result in the degradation of RNA.

8.3 Peripheral Blood Mononuclear Cell (PBMC) Cryopreservation

The PBMCs are isolated from whole blood collected in EDTA, ACD, or heparin tubes using density gradient centrifugation. Cell separation is followed by a check for cell viability and number of cells isolated from the patient specimen. Cell numbers are adjusted such that each aliquot contains from 100 000 to 5 000 000 cells per aliquot. Yield of PBMCs depends on each patient's WBC count and differential. A WBC count of 5000 with 40% lymphs yields approximately 2 x 10^6 cells per mL of whole blood, since density gradient centrifugation essentially eliminates neutrophils. For unknown cell counts, typically 1 000 000 cells (primarily lymphocytes) can be recovered from a milliliter of whole blood after density gradient separation. Cells are aliquoted into polyethylene vials designed for low temperature storage and then can be transferred into plastic freezing containers and placed in a -70 ^{o}C or lower freezer, depending on how long samples need to be stored in a repository.

Density-gradient-separated peripheral blood mononuclear cells (PBMC) are cryopreserved for future analysis. The following is an example of a validated procedure for cryopreservation and thawing of PBMC to perform *in vitro* functional analysis. It is important that cryopreservation procedures are validated and compared with performance of fresh PBMC. It is recommended that 80% viability and 75% recoveries are achieved with cryopreserved samples to be used in *in vitro* functional studies. The following protocol for freezing PBMC uses a final concentration of 10% dimethylsulfoxide (DMSO) and 11.25% protein (human serum albumin) in RPMI. Cryoprotectants, such as DMSO, reduce the amount of ice formed during freezing and reduce solute concentration, thus reducing ionic stress. However, these compounds can themselves cause osmotic injury during addition or removal, since they are hypertonic. It is therefore important to follow procedures to minimize these effects.

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Appendix A. Disruption of Tissue Samples Using Mortar and Pestle

(1) Freeze the sample in liquid nitrogen immediately after harvesting. Do not allow the sample to thaw at any time during disruption.

(2) Precool the mortar to -20 °C and keep it on dry ice.

(3) Pour liquid nitrogen into the mortar, and precool the pestle by placing the grinding end in the liquid nitrogen.

(4) Place the frozen tissue in the mortar and grind until a fine, whitish powder is formed. Add liquid nitrogen as necessary during the grinding.

(5) Transfer the powder using a precooled spatula to precooled containers of the appropriate size.

(6) Allow the liquid nitrogen to evaporate. To prevent the sample from thawing after the liquid nitrogen has evaporated, the container should be cooled by placing it in dry ice or liquid nitrogen.

(7) Add the lysis buffer as quickly as possible and continue with the isolation procedure.

For fixed tissues, the fixative should be removed prior to lysis. Formalin can be removed by washing the tissue in PBS. Paraffin should similarly be removed from paraffin-embedded tissues by extraction with xylene followed by washing with ethanol.

Appendix B. PBMC Cryopreservation and Thawing Procedure

The following equipment/supplies should be used:

- sterile, 50-mL polypropylene, conical tubes;
- 200-µL micropipettor;
- serological pipettor;
- microscope for cell counting;
- 37 °C water bath;
- sterile, disposable 5-mL and 10-mL serological pipette;
- 1.8- and/or 3.6-mL cryovials;
- -70 °C or lower freezer;
- plastic/polycarbonate freezing containers;
- liquid nitrogen freezer; and
- hemacytometer.

The following reagents should be used:

- *Stock 25% human serum albumin (HSA)* — Prepare 25% HSA in RPMI (e.g., dissolve 25 g human albumin fraction V into 100 mL RPMI-1640 [not cRPMI]). Allow 24 hours to completely dissolve. Store at 4 ^{o}C.

- *12.5% human serum albumin* — Combine 10 mL of stock 25% HSA and 10 mL of sterile RPMI-1640. Store at 4 ^{o}C.

- *2x freezing medium* — Combine 10 mL of stock 25% HSA and 10 mL of sterile RPMI-1640 medium. Add 5 mL of DMSO. Store at 4 ^{o}C for up to 30 days.

- *Trypan blue* — Prepare 0.4% trypan blue in PBS.

- *RPMI medium for cell culture (cRPMI)* — Supplement sterile RPMI-1640 medium with 10% sterile, heat-inactivated FCS and 1% sterile antibiotic/antimycotic. Store at 4 ^{o}C.

Freezing PBMC

(1) Label cryovials with: patient ID; 10 x 10^6 PBMC; the blood draw date; and the technologist's initials.

(2) Place labeled cryovials in -20 °C freezer.

(3) Resuspend PBMC at 1 x 10^7 viable lymphocytes/mL in 12.5% HSA/RPMI prechilled to 4 ^{o}C in a 50-mL conical polypropylene tube.

(4) While *gently* swirling the tube, add dropwise enough 4 ^{o}C 2x freezing medium to double the volume of the cell suspension (e.g., if 10 mL of cell suspension, add 10 mL of 2x freezing medium).

(5) Immediately place tube on ice. Avoid any further mixing or agitation of cells. Slowly aspirate the cell suspension into a pipette and dispense 1 mL per cryovial on ice.

(6) Place cryovials in a precooled plastic/polycarbonate freezing container prefilled with 70% isopropanol according to the manufacturer's instructions. Place the freezing container at -70 ^{o}C or lower.

Appendix B. (Continued)

(7) For each cryovial, 1 mL of total volume is added per 10 x 10^6 PBMC.

(8) Prepare 2x freezing media. For 20 cryovials, prepare 10 mL 12.5% HSA solution, and 2.5 mL DMSO. Mix and place on ice for a minimum of 30 minutes.

(9) For long-term storage, transfer the cryovials into a liquid nitrogen freezer after 24 hours of freezing at -70 °C or lower. Never store cells at -20 °C, even temporarily.

(10) Record the vial location (box number and slot number) on both the specimen database and the freezer binder.

Although a number of freezing, thawing, and shipping protocols have been adopted for preservation of clinical samples for subsequent evaluation of leukocyte phenotype and function, it is important to validate the process for a particular application when selecting a protocol and to maintain minimal cellular viability and recoveries. T-lymphocyte function depends on not only viability and recovery of the T-subset, but also the ability of antigen-presenting cells to present cognate antigen. Therefore, in addition, functional responses should also be validated. It is recommended that cell viability be 80% and cell recoveries maintained above 75%. Functional responses (i.e., % cytokine positive cells) of cryopreserved PBMC should not vary from those values obtained with fresh samples by more than 10%.

Thawing PBMC

(1) Place the cell culture media in a 37 °C water bath for about 30 minutes.

(2) Label a 50-mL centrifuge tube per sample and add 8 mL of warm (22 °C to 37 °C) assay media.

(3) Remove cryovials from the liquid nitrogen freezer and place directly on dry ice.

(4) Thaw no more than two cryovials at a time. Place cryovials in a 37 °C water bath until cell suspension is almost completely melted or a small bit of ice remains.

(5) Dry off the outside of the cryovials and wipe with 70% ethanol, taking care not to remove the sample identification.

(6) Add slowly 1 mL of warm (22 °C to 37 °C) cell culture media to the thawed cells.

(7) Transfer the cell suspension to the 50-mL polypropylene tubes containing 8 mL of media.

(8) Balance the tubes and centrifuge at 1200 rpm for ten minutes.

(9) Aspirate the supernatant and resuspend the cells in 2 mL assay medium.

(10) Perform the manual cell count using trypan blue to determine PBMC viability.

A standard thawing procedure is equally as or perhaps more important for obtaining maximum viability and recoveries of cryopreserved PBMC. The thawing procedure should also become part of the validation exercise to ensure reproducible sample preparation. Generally, it is recommended that prior to thawing, cell culture media is prewarmed for 30 minutes at 37 ºC and 8 mL aliquoted into 50-mL centrifuge tubes for each cryovial.

Clinical and Laboratory Standards Institute consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact CLSI or visit our website at www.clsi.org.

Summary of Consensus/Delegate Comments and Subcommittee Responses

MM13-P: *Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Proposed Guideline*

General

1. This seems comprehensive, but it would be more useful if it told you where to find methods for "measurand quality assessment," since they are not included in this document.

- **When the analytical method requires quality assessment of the measurand (purified DNA or RNA), please refer to the following CLSI/NCCLS documents: MM3—*Molecular Diagnostic Methods for Infectious Diseases*, MM5—*Nucleic Acid Amplification Assays for Molecular Hematopathology*, or MM9—*Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine*. Otherwise, refer to the manufacturer's recommendations. The note at the end of the Scope has been revised to refer the reader to the documents listed above.**

Section 6.2, General Recommendations for Storage of Purified DNA

2. There is a small inconsistency between Section 6.2 and Section 8.1. Section 6.2 states that purified DNA can be stored "at 2 to 8 ˚C for at least one year if contaminating DNases are absent," whereas Section 8.1 states that "Once isolated, DNA is stable at 2 to 8 °C for at least three years." I think this should be rewritten so as to be consistent.

- **The text has been revised for consistency. The recommendation of three years has been revised to "at least one year" in Section 8.1. This is based on the reference given in Section 6.2.**

Section 6.4.1, Whole Blood, Serum, and Plasma

3. Rewrite the last sentence on the page to say, "extraction should begin *within four hours"* instead of "within one to four hours." The latter implies that one has to wait at least one hour before beginning an extraction.

- **The text has been revised as suggested.**

Section 6.4.2, Bronchoalveolar Lavage (BAL) and Section 6.4.13, Sputum

4. A consensus can be made about the temperature of an ultralow freezer. In the document, it is sometimes expressed as -70 °C (Section 6.4.13), -72 °C (Section 6.4.2), or -80 °C.

- **Where appropriate, the document has been revised to recommend a storage temperature of -70 °C or lower.**

Section 6.4.5, Buffy Coat

5. In the first sentence in the paragraph, I think the phrase "a few days" is too vague. Specify how many days after collection that whole blood can be stored before DNA must be extracted.

- **The subcommittee agrees and the text has been revised to recommend three days as found in the reference cited.**

Section 6.4.6.1, DNA

6. It states that "CSF specimens for HSV, CMV, EBV, and VZV should be placed at -20 to -80 °C, implying that CSF specimens for all other organisms do not need to be subjected to these storage conditions. Maybe remove the mention of specific viruses and leave it generic.

- **The text has been revised as follows: "If specimens cannot be processed immediately, CSF specimens tested for DNA viruses (e.g., HSV, CMV, EBV, and VZV) should be placed at -20 or -70 °C or lower."**

Section 6.4.9.2, RNA and Section 8.2, Sample Storage Requirements - RNA

7. Storage of RNA (Sections 6.4.9.2 and 8.2)—common commercial kits available for purification of RNA use silica-based technology on columns, and no precipitation step using ethanol is required after elution. Therefore, it may not be appropriate to add ethanol to these samples for short- or long-term storage at -80 °C.

- **Following purification, kit manufacturer's instructions may not always be applicable. Whether ethanol is added is based on the downstream analytical method.**

Section 6.4.15, Cervical and Urethral Swabs

8. The fourth sentence in the paragraph states, "One milliliter of this fluid is either stored..." I feel this is too specific, as some labs may choose to store more and sometimes are forced to only store less. Maybe change to "An appropriate aliquot."

- **The text has been revised as follows: "For further testing, a required amount of the transport fluid may be either stored at -70 ºC or lower or immediately centrifuged, and the pellet processed for DNA or RNA according to the assay manufacturer's recommendations."**

Section 7.3.2.6.1.3, Disruption of Tissue Samples Using a Mortar and Pestle

9. I think there is too much methodological detail here, describing exactly how a mortar and pestle is used. Section 6.3.3.1.3 covers exactly the same ground (except for use on RNA), but does so in a more appropriate manner without all the methodological details. Perhaps the former section could be rewritten in a more generic manner.

- **The subcommittee agrees that the detailed protocol does not need to appear in this section; however, the subcommittee believes that the detailed protocol is useful and has moved the protocol to Appendix A.**

Section 7.3.2.9, Isolation of DNA From Pathogens

10. Last sentence: I have never heard of the practice of treating swab samples with a fungicide before centrifugation of bacteria, if the intent is simply to isolate DNA from cells. Maybe if the intent is to culture the bacteria, then a fungicide is appropriate, but I don't see how it benefits DNA extraction. Perhaps I am wrong about this.

- **The last sentence recommending the pretreatment of swabs with a fungicide has been deleted.**

11. Section 7.3.3.2.1, Enzymatic Digestion

    This method cannot be used with tissues since lysis with proteinase K is relatively slow and inefficient, and it is difficult to prevent endogenous RNases from degrading the RNA and Section 7.3.3.3.1 heart, muscle, and skin tissue " ...In order to remove these proteins, which can interfere with RNA isolation, the sample needs to be treated with a protease. However, the protease digest needs to be carried out under conditions that do not allow RNA degradation."

    The above two phrases highlight the need for removing proteins from the tissue. The usage of protease is first suggested, followed by saying that proteinase K is not recommended. The document still does not provide readers with a proper method to deal with the issue.

- **The following text has been added to the end of the first paragraph of Section 7.3.3.2.1:**

  "Alternatively, a disruption under chaotropic conditions can be performed; subsequent dilution of the lysate to a salt concentration tolerated by enzymes like proteinase K can increase the yield of RNA without degradation during the enzymatic treatment."

Section 8, Sample Storage

12. I think it would be useful to include a separate subsection that provides guidelines as to the proper form of documentation or cataloguing of stored nucleic acids. Many of us are in the process of establishing formal "DNA banks" or are otherwise concerned about record keeping as it pertains to stored DNA or RNA and would like guidelines as to how proper records and other documentation can be kept (e.g., forms of identifiers, electronic vs. hard copies, information backup, type of information to be included on the tube label, coding practices). Maybe it would suffice to say that documentation of stored DNA/RNA should follow the same practices as for any other biological specimen (serum, tissue, etc.), but then again, maybe there are some special requirements. Perhaps this is somewhat naïve, but I personally don't know what the best document format would be.

- **There are no standardized recommendations at this time and it is beyond the scope of this document to establish such guidelines.**

The Quality System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care.* The quality management system approach applies a core set of "quality system essentials" (QSEs), basic to any organization, to all operations in any healthcare service's path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

MM13-A addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X MM3 MM5 MM9						M29

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

MM13-A addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

Preexamination				**Examination**			**Postexamination**	
Examination ordering	Sample collection	Sample transport	Sample receipt/processing	Examination	Results review and follow-up	Interpretation	Results reporting and archiving	Sample management
	X LA4 MM5 MM9	X LA4	X LA4	MM3 MM5 MM9		MM3 MM5 MM9	MM3 MM5 MM9	X LA4

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

Related CLSI/NCCLS Publications*

LA4-A4 **Blood Collection on Filter Paper for Newborn Screening Programs; Approved Standard—Fourth Edition (2003).** This document addresses the issues associated with specimen collection, the filter paper collection device, and the transfer of blood onto filter paper, and provides uniform techniques for collecting the best possible specimen for use in newborn screening programs.

M29-A3 **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on U.S. regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

MM3-P2 **Molecular Diagnostic Methods for Infectious Diseases; Proposed Guideline—Second Edition (2005).** This guideline addresses topics relating to clinical applications, amplified and nonamplified nucleic acid methods, selection and qualification of nucleic acid sequences, establishment and evaluation of test performance characteristics, inhibitors, and interfering substances, controlling false-positive reactions, reporting and interpretation of results, quality assurance, regulatory issues, and recommendations for manufacturers and clinical laboratories.

MM5-A **Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline (2003).** This guideline addresses the performance and application of assays for gene rearrangement and translocations by both polymerase chain reaction (PCR) and reverse-transcriptase polymerase chain reaction (RT-PCR) techniques and includes information on specimen collection, sample preparation, test reporting, test validation, and quality assurance.

MM9-A **Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline (2004).** This document addresses automated, PCR-based, dideoxy-terminator, and primer extension sequencing done on gel- or capillary-based sequencers. Topics covered include: specimen collection and handling; isolation of nucleic acid; amplification and sequencing of nucleic acids; interpretation and reporting of results; and quality control/assessment considerations as appropriate.

* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most current editions.

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